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(54) **METHOD FOR SCREENING A MODULATOR OF A TMEM16 FAMILY MEMBER**

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PCT Pub. Date: **Oct. 24, 2013**

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US 2015/0079599 A1 Mar. 19, 2015

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(51) **Int. Cl.**

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G01N 33/50	(2006.01)
C12Q 1/34	(2006.01)
G01N 33/92	(2006.01)

(52) **U.S. Cl.**

CPC ***G01N 33/5023*** (2013.01); ***C12Q 1/34*** (2013.01); ***G01N 33/5008*** (2013.01); ***G01N 33/92*** (2013.01); *G01N 2500/00* (2013.01); *G01N 2500/04* (2013.01)

(58) **Field of Classification Search**

CPC C12Q 1/34; G01N 33/5008; G01N 2500/00; G01N 33/5023; G01N 2500/04; G01N 33/92

See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to a method for screening a modulator of a TMEM16 family member, which comprises the following steps:

(1) treating cells expressing the TMEM16 family member with a candidate of the modulator, and

(2) determining whether the candidate alters distribution of a lipid selected from phosphatidylserine, phosphatidylcholine, and galactosylceramide in plasma membrane of the cells,

wherein a candidate which increases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member, and

a candidate which increases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member.

5 Claims, 29 Drawing Sheets

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Fig. 1A

A

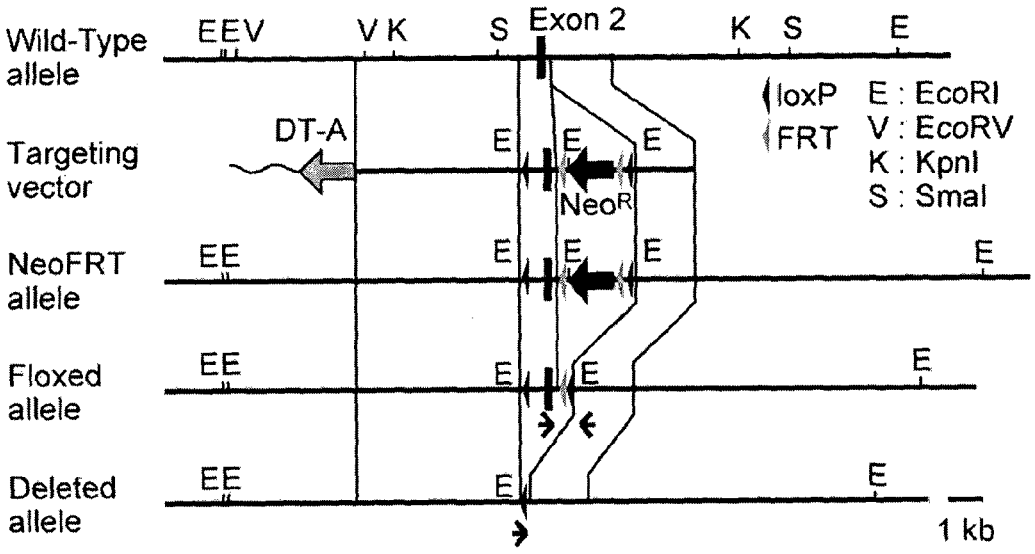

Wild-Type allele
Targeting vector
NeoFRT allele
Floxed allele
Deleted allele
EEV
V K
S
Exon 2
K S
E
DT-A
NeoR
EE
loxP
FRT
E : EcoRI
V : EcoRV
K : KpnI
S : SmaI
1 kb

Fig. 1B

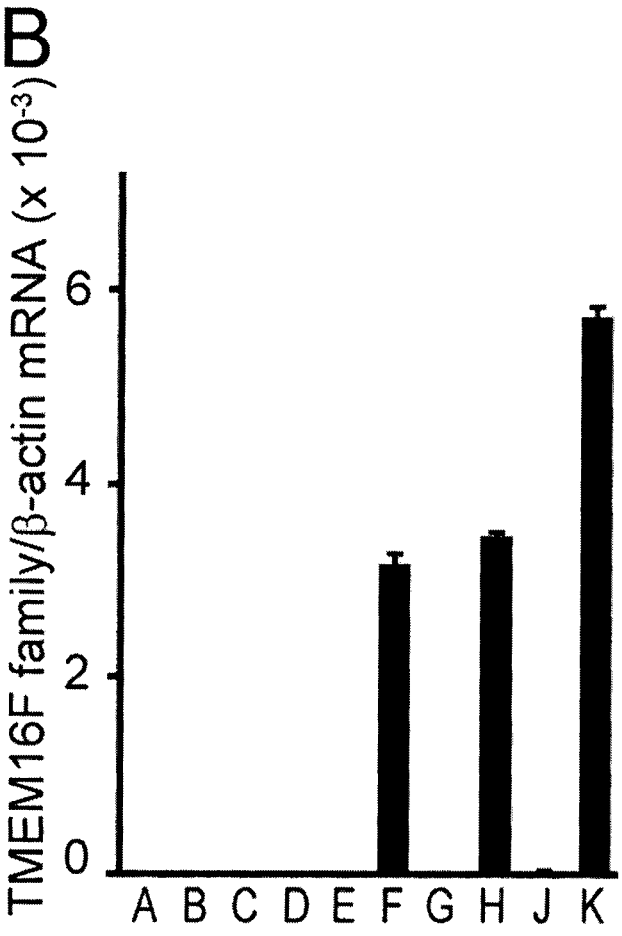


Fig. 1C

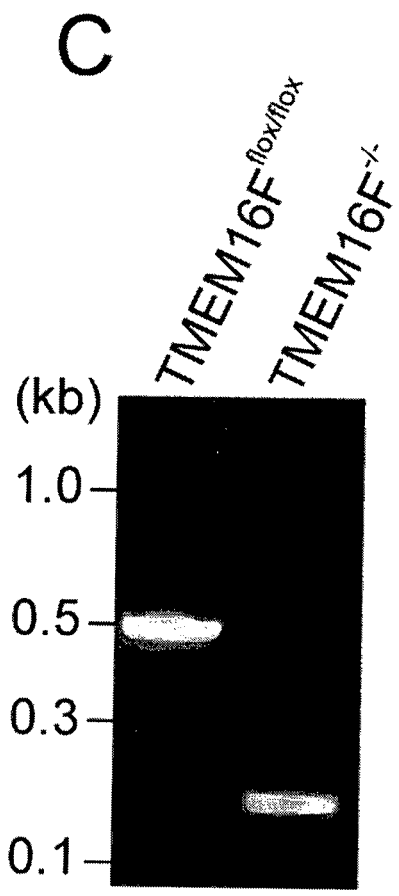


Fig. 1D

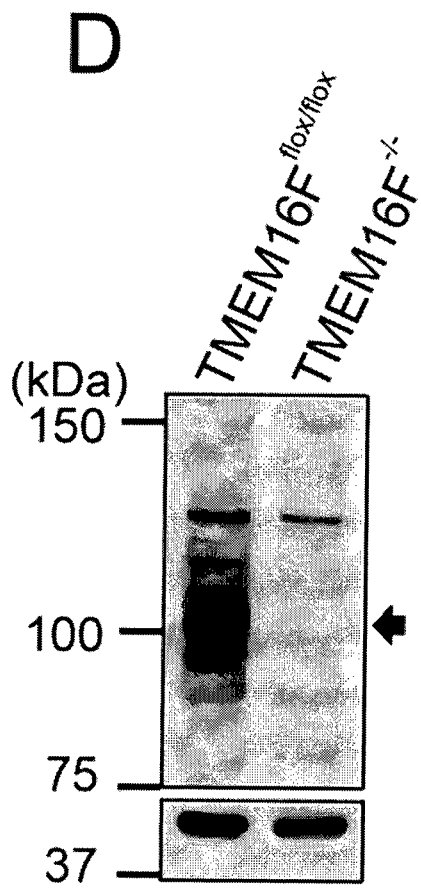


Fig. 2A

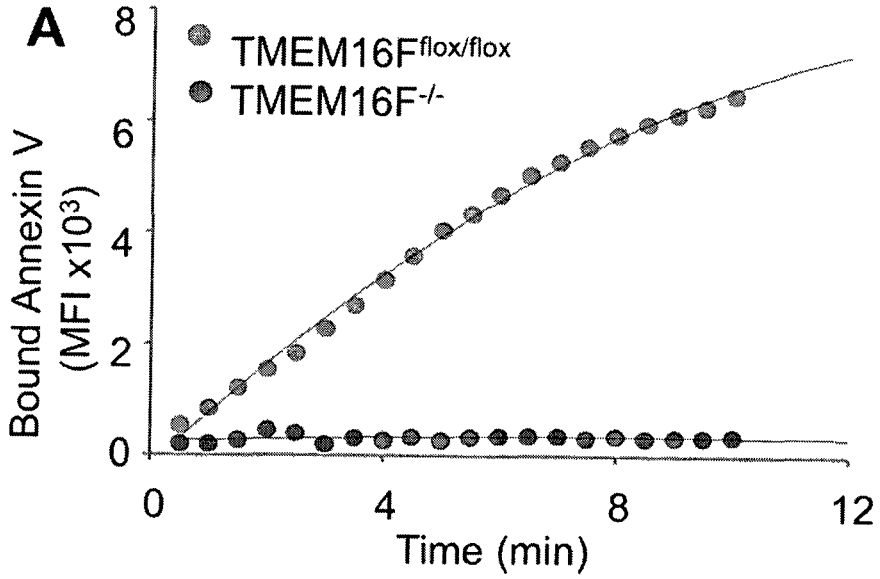

A
TMEM16F flox/flox
TMEM16F -/-
Bound Annexin V (MFI x10³)
0
2
4
6
8
0
4
8
12
Time (min)

Fig. 2B

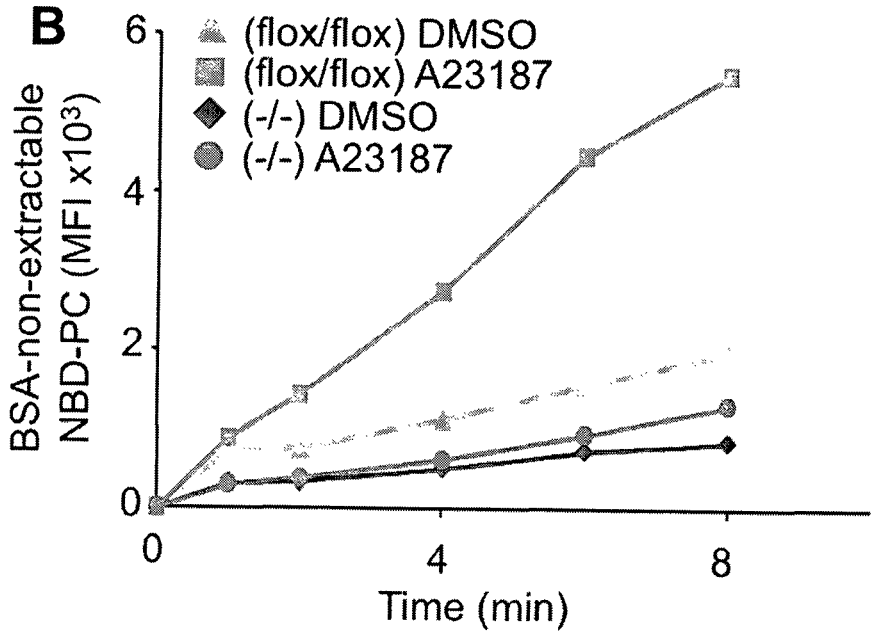

B
(flox/flox) DMSO
(flox/flox) A23187
(-/-) DMSO
(-/-) A23187
BSA-non-extractable NBD-PC (MFI x10³)
0
2
4
6
0
4
8
Time (min)

Fig. 2C

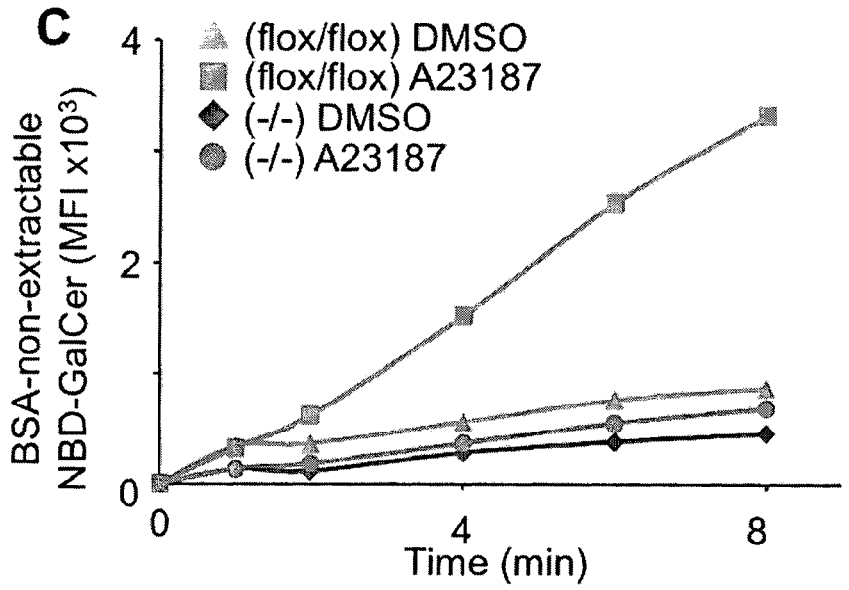

C
(flox/flox) DMSO
(flox/flox) A23187
(-/-) DMSO
(-/-) A23187
BSA-non-extractable NBD-GalCer (MFI x10³)
4
2
0
0
4
8
Time (min)

Fig. 2D

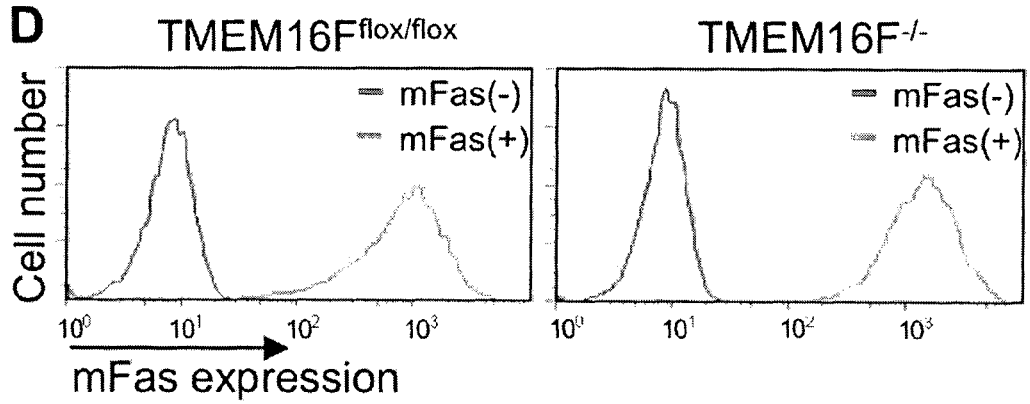

D
TMEM16F flox/flox
TMEM16F -/-
mFas(-)
mFas(+)
Cell number
10⁰
10¹
10²
10³
mFas expression

Fig. 2E

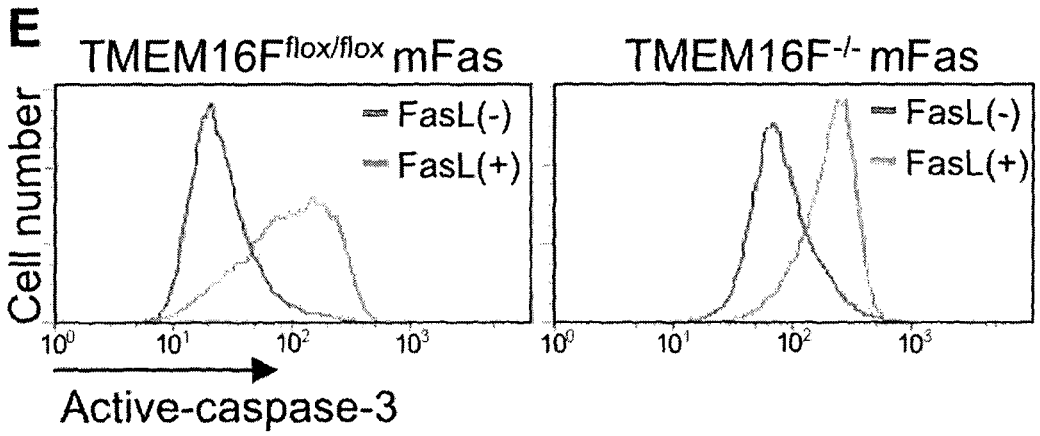
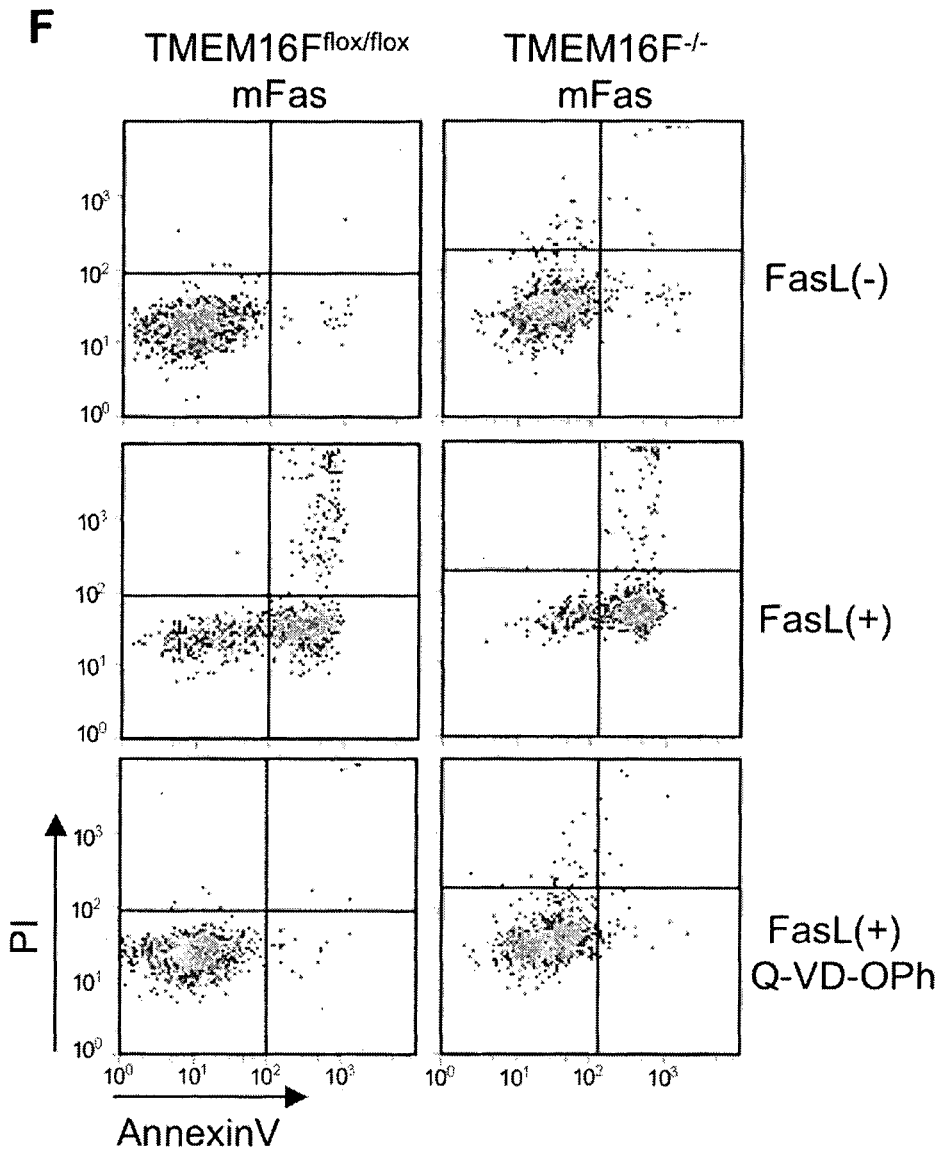

E
TMEM16F flox/flox mFas
TMEM16F -/- mFas
FasL(-)
FasL(+)
Cell number
10^0
10^1
10^2
10^3
Active-caspase-3

Fig. 2F


F
TMEM16F flox/flox
mFas
TMEM16F -/-
mFas
FasL(-)
FasL(+)
FasL(+)
Q-VD-OPh
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AnnexinV
10^0
10^1
10^2
10^3

Fig. 2G

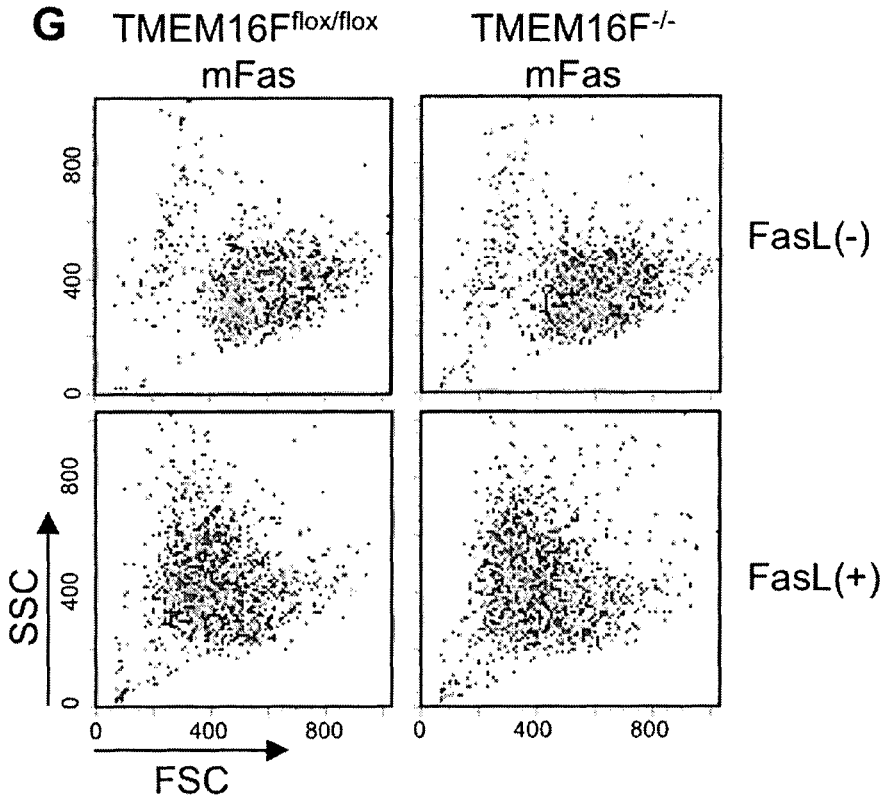
G
TMEM16F flox/flox
mFas
TMEM16F -/-
mFas
FasL(-)
FasL(+)
SSC
FSC
0
400
800

Fig. 3A

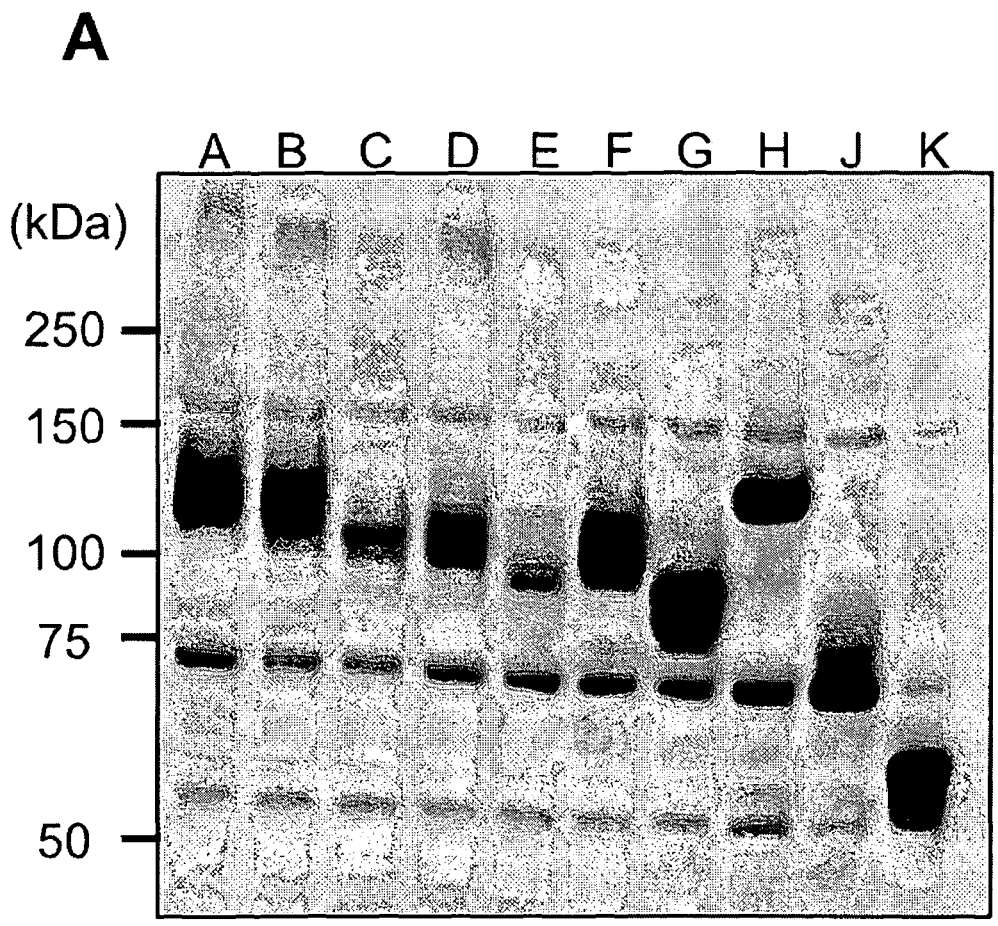

Fig. 3B

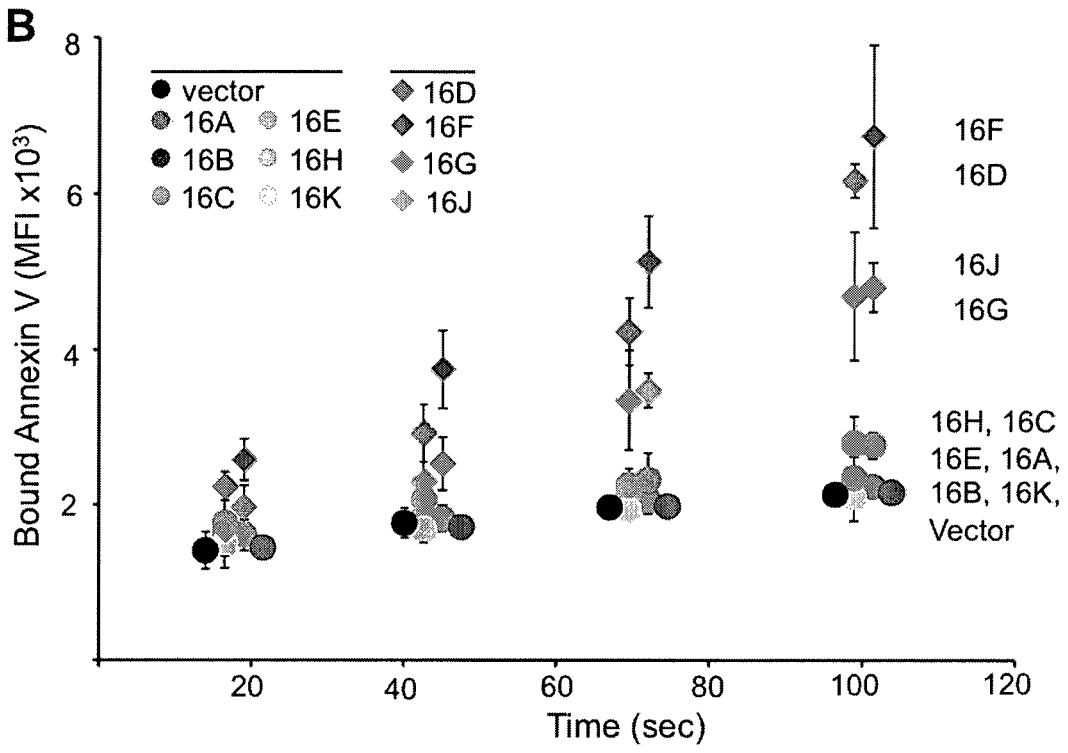
B
Bound Annexin V (MFI x10³)
8
6
4
2
vector
16A
16B
16C
16E
16H
16K
16D
16F
16G
16J
16F
16D
16J
16G
16H, 16C
16E, 16A,
16B, 16K,
Vector
20
40
60
80
100
120
Time (sec)

Fig. 4A

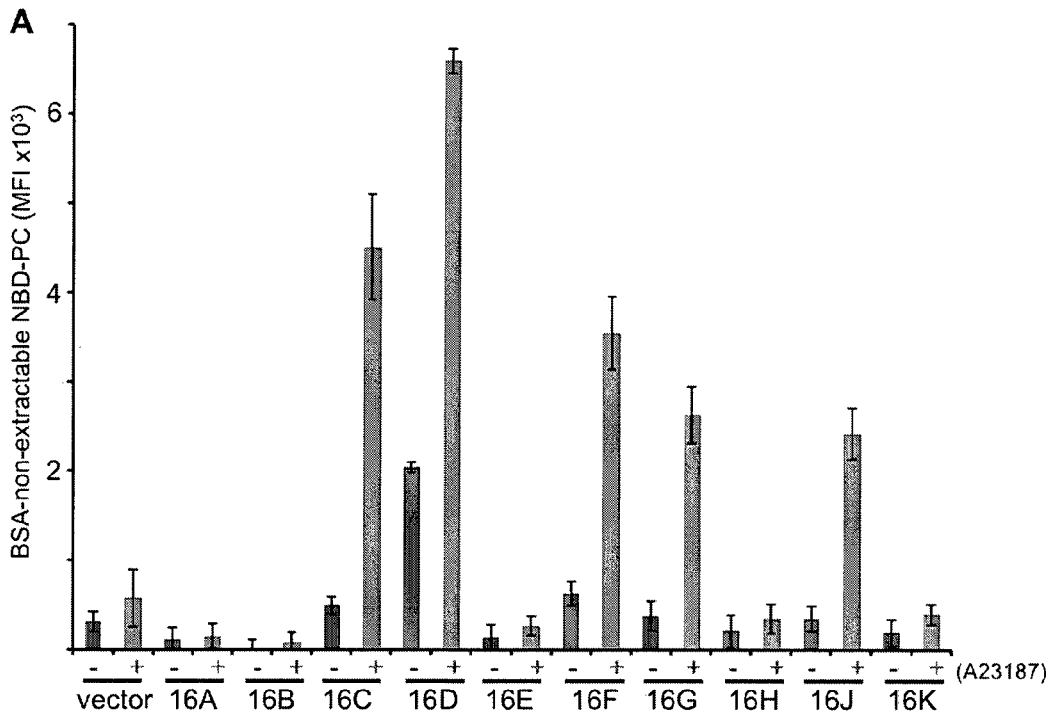
A
BSA-non-extractable NBD-PC (MFI x10³)
6
4
2
- + - + - + - + - + - + - + - + - + - + - +
(A23187)
vector
16A
16B
16C
16D
16E
16F
16G
16H
16J
16K

Fig. 4B

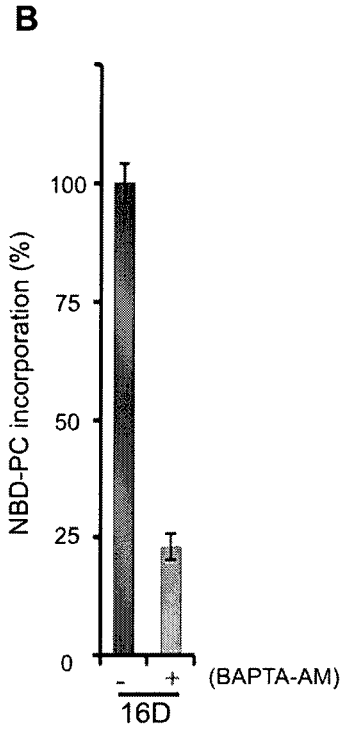

Fig. 4C

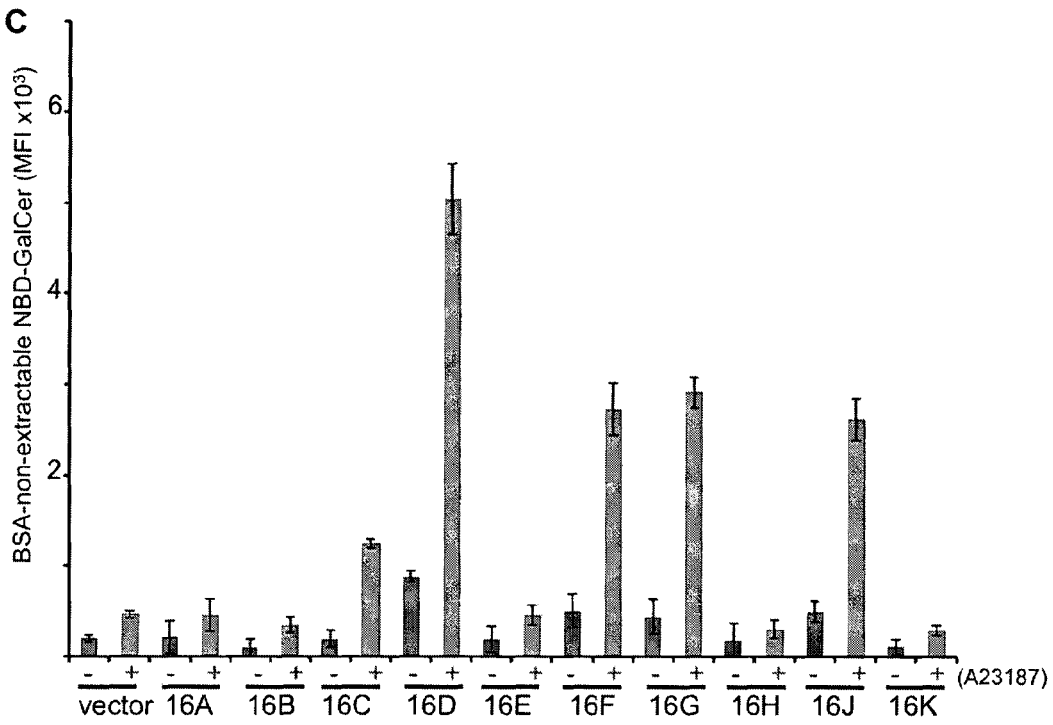
C
BSA-non-extractable NBD-GalCer (MFI x10³)
6
4
2
- + - + - + - + - + - + - + - + - + - + - + (A23187)
vector 16A 16B 16C 16D 16E 16F 16G 16H 16J 16K

Fig. 5A

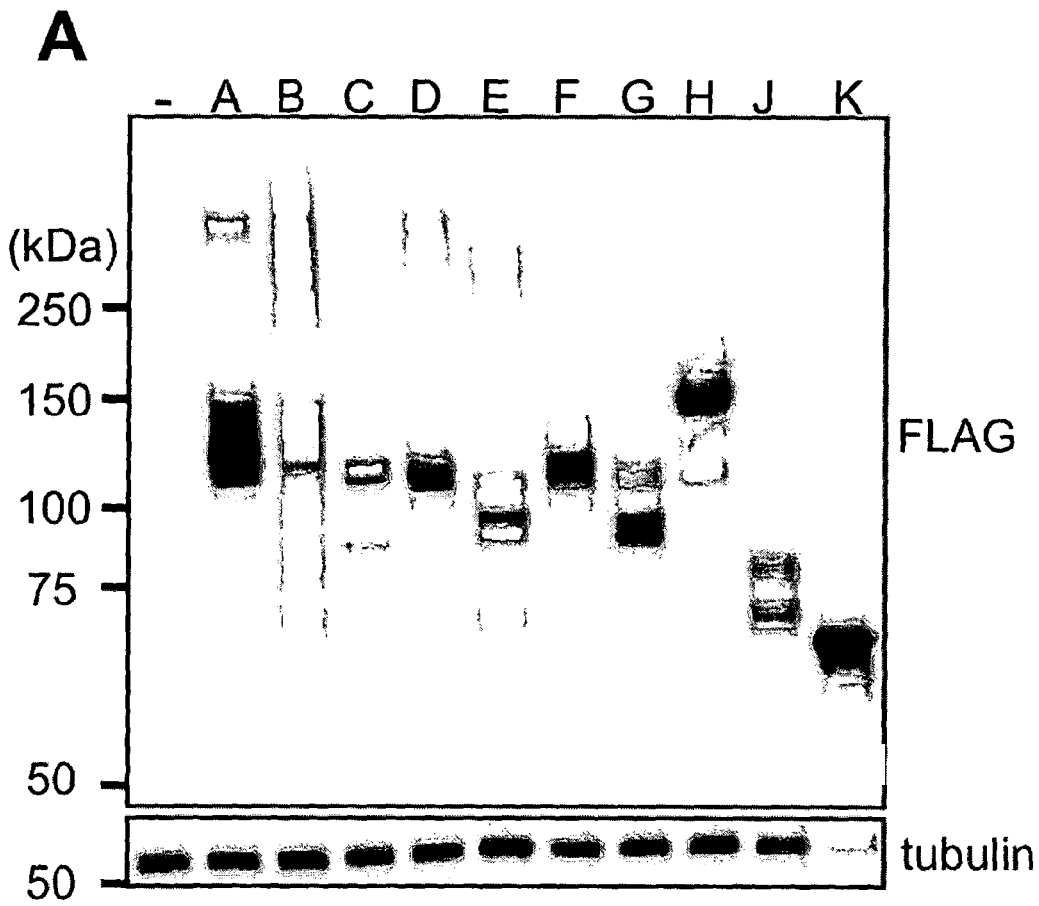

Fig. 5B

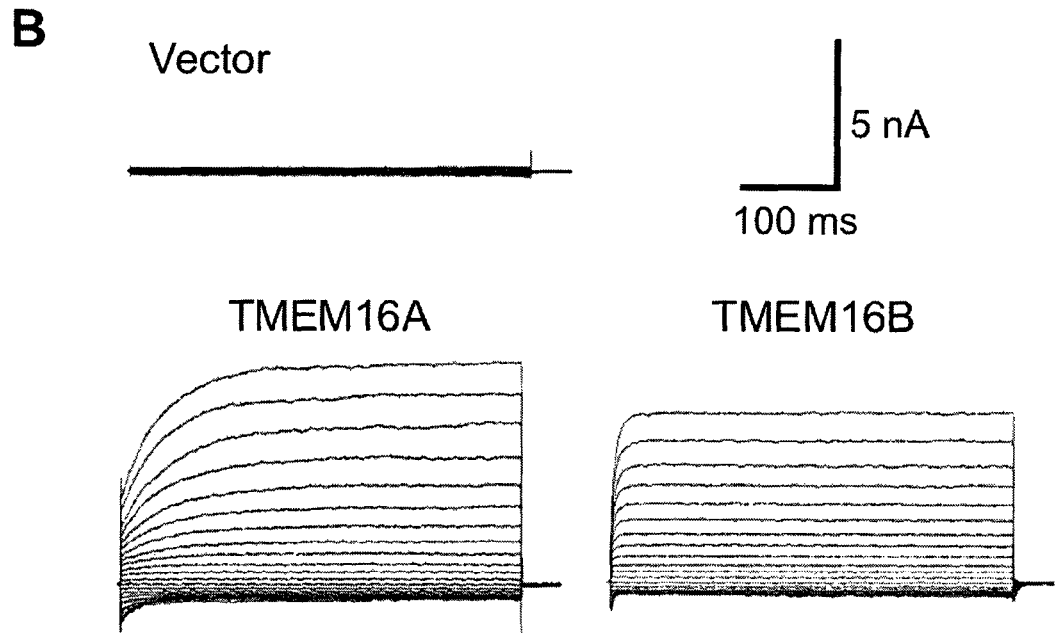

Fig. 5C

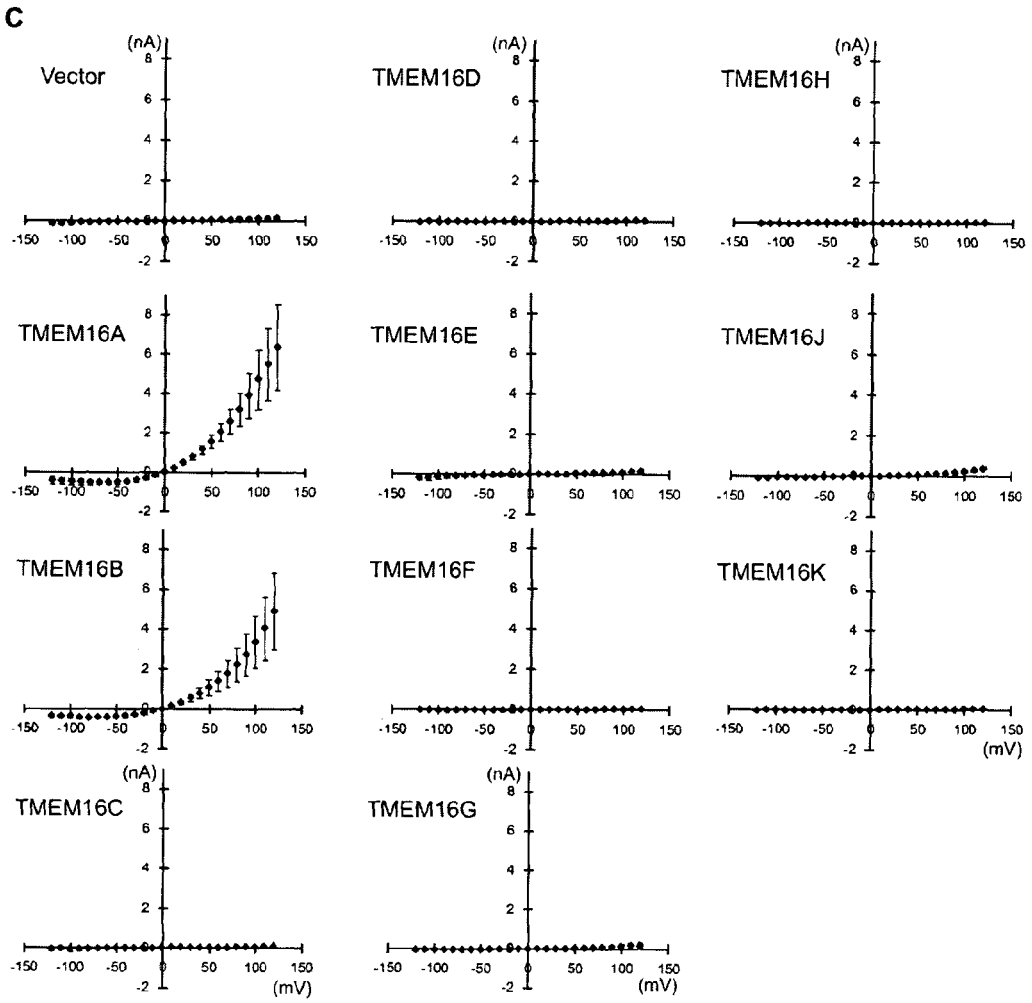
C
Vector
TMEM16D
TMEM16H
TMEM16A
TMEM16E
TMEM16J
TMEM16B
TMEM16F
TMEM16K
TMEM16C
TMEM16G
(nA)
(mV)

Fig. 6A

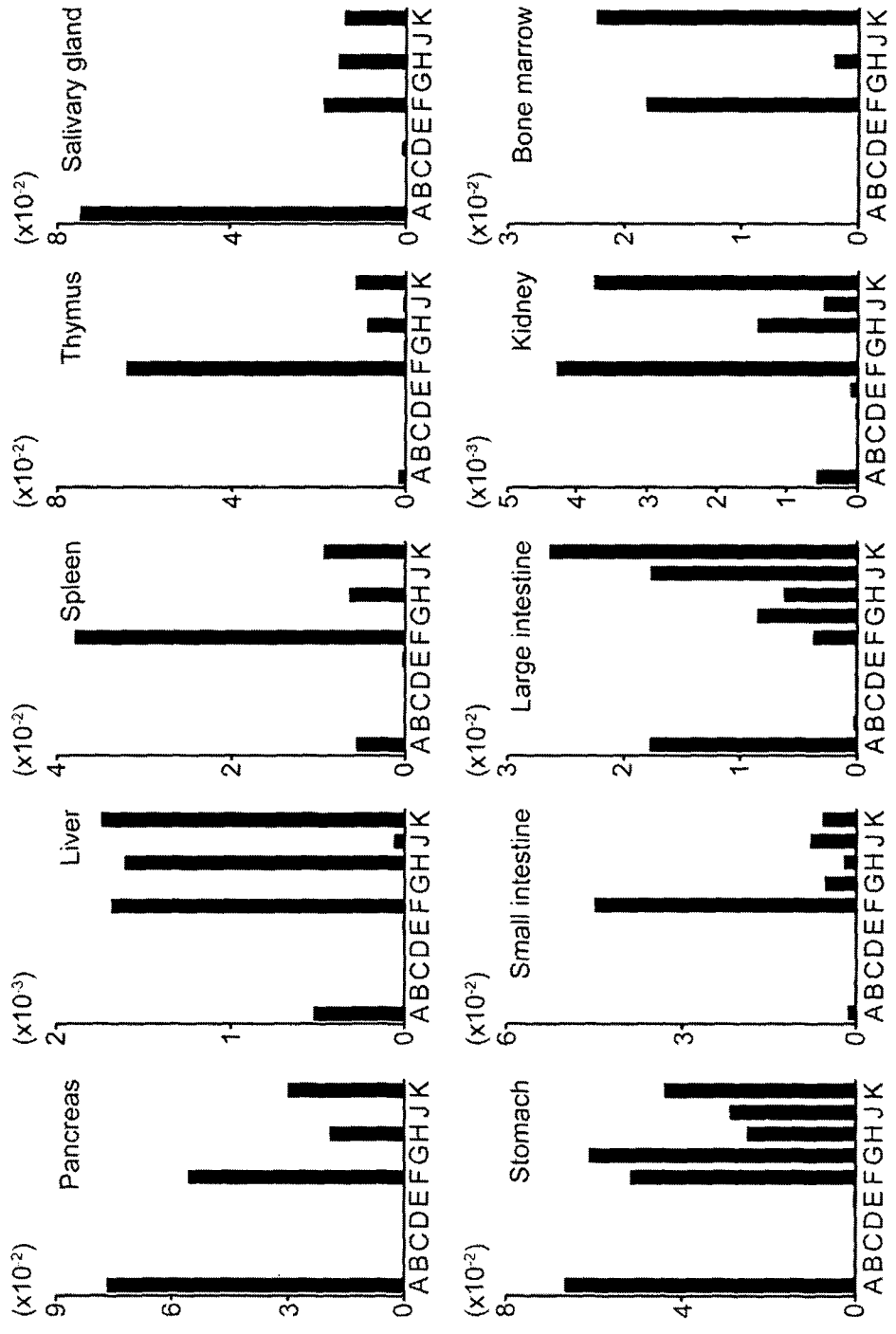
Pancreas
Liver
Spleen
Thymus
Salivary gland
Stomach
Small intestine
Large intestine
Kidney
Bone marrow
(x10-2)
(x10-3)
A B C D E F G H J K
TMEM16 family / β-actin mRNA

Fig. 6B

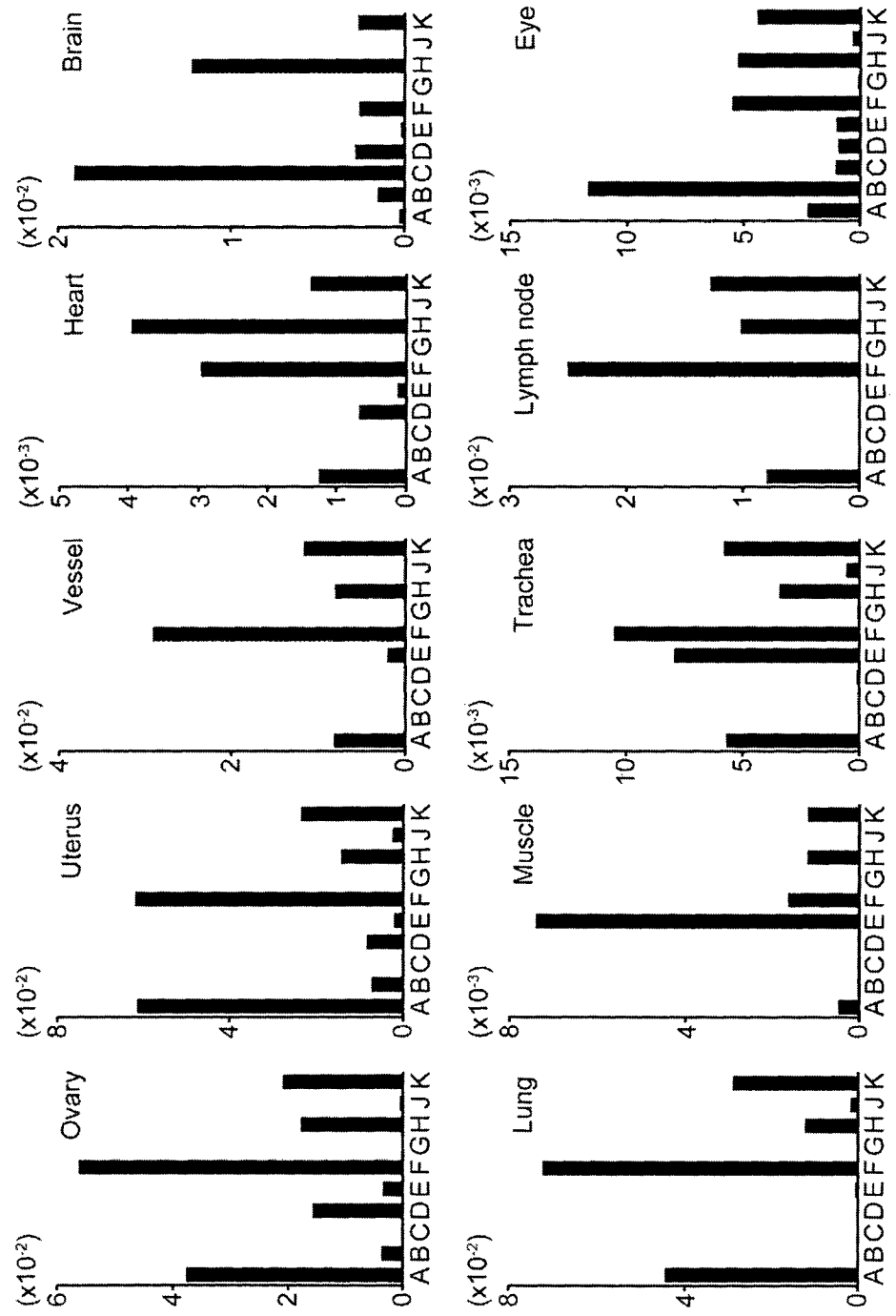
Ovary
Uterus
Vessel
Heart
Brain
Lung
Muscle
Trachea
Lymph node
Eye
(x10-2)
(x10-3)
ABCDEFGHIJK
TMEM16 family / β-actin mRNA

Fig. 6C

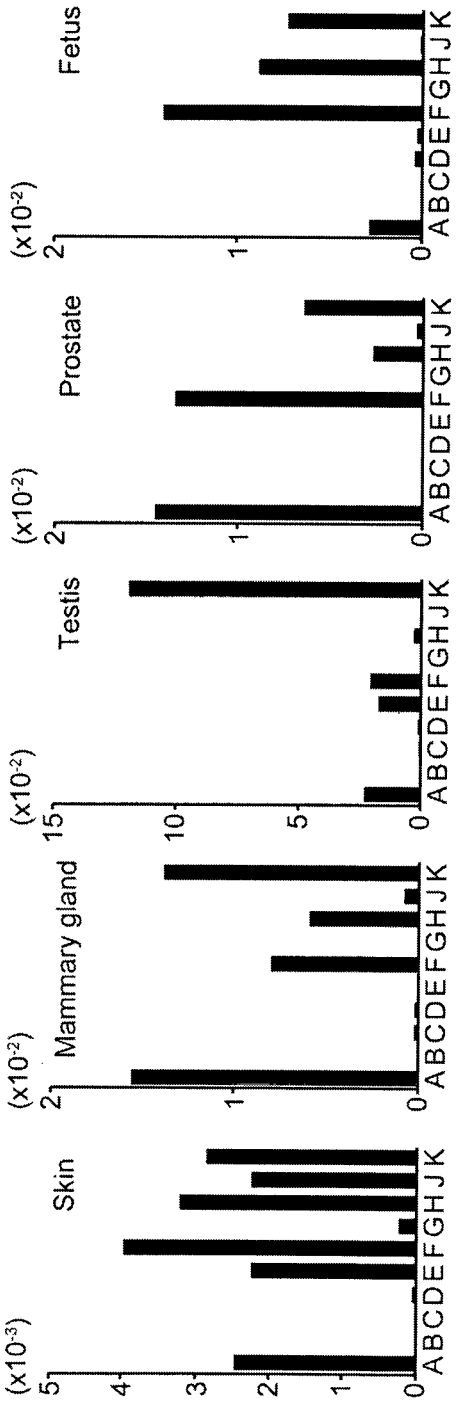
Skin
Mammary gland
Testis
Prostate
Fetus
(x10-3)
(x10-2)
ABCDEFGHJK
TMEM16 family / β-actin mRNA

Fig. 7A

```
        10          20          30          40          50          60
GGCGCGCCGG ATCCGCCACC ATGGTGCACC ACAGCGGCAG CATCCAGAGC TTCAAGCAGC

        70          80          90         100         110         120
AGAAAGGCAT GAACATCAGC AAGAGCGAGA TCACCACCGA GGCCAGCCTG AAGCCCAGCA

       130         140         150         160         170         180
GAAGAAGCCT GCCCTGCCTG GCCCAGAGCT ACGCCCACAG CAAGAGCCTG AGCCAGAGCG

       190         200         210         220         230         240
CCAGCCTGTT CCAGAGCACC GAGAGCGAGA GCCAGGCCCC TACCAGCGTG ACCTTCCTGA

       250         260         270         280         290         300
GCGCCGACAA GCCCGAGCAC GTGACCAGCG AGGAAAGCAG AAAGGACAGC ACCCTGAAGT

       310         320         330         340         350         360
GCAGCTTCGC CGACCTGAGC GACTTCTGTC TGGCCCTGGG CAAGGACAAG GACTACCTGG

       370         380         390         400         410         420
ACGAGAGCGA GCACGCCAAC TACGACAGAA GCAGACTGCT GAACGACTTC GTGACCAAGG

       430         440         450         460         470         480
ACAAGCCCGC CAGCAAGACC AAGCTGAGCA AGAACGACAT GAGCTATATC GCCAGCAGCG

       490         500         510         520         530         540
GCCTGCTGTT CAAGGACGGC AAGAAGAGAA TCGACTACAT CCTGGTGTAC CGCAAGACCA

       550         560         570         580         590         600
ACATCCAGTA CGACAAGAGG AACACCTTCG AGAAGAACCT GAGAGCCGAG GGCCTGATGC

       610         620         630         640         650         660
TGGAAAAAGA GCCCGCTATC GCCAACCCCG ACATCATGTT TATCAAGATC CACATCCCCT

       670         680         690         700         710         720
GGGACACCCT GTGCAAATAC GCCGAGAGAC TGAACATCAG GGTGCCCTTC CGGAAGAAGT

       730         740         750         760         770         780
GCTACTACAC CGACCAGAAG AACAAGAGCA AGAGCAGGGT GCAGAACTAC TTCAAGCGGA

       790         800         810         820         830         840
TCAAGAAATG GATGAGCCAG AACCCCATGG TGCTGGACAA GAGCGCCTTC CCCGAGCTGG

       850         860         870         880         890         900
AAGAGAGCGA CTGCTACACC GGCCCCTTCA GCAGAGCCAG AATCCACCAC TTCATCATCA

       910         920         930         940         950         960
ACAACAAGGA CACCTTCTTC AGCAACGCCA CCAGATCCAG AATCGTGTAC CACATGCTGG

       970         980         990        1000        1010        1020
AACGGACTAA GTACGAGAAC GGCATCAGCA AAGTGGGCAT CAGAAAGCTG ATCACCAACG

      1030        1040        1050        1060        1070        1080
GCTCCTATAT CGCCGCCTTC CCACCCCACG AGGGCGCCTA CAAGAGCAGC CTGCCCATCA
```

Fig. 7B

```
      1090       1100       1110       1120       1130       1140
AGACCCACGG CCCCCAGAAC AACAGACATC TGCTGTACGA GAGATGGGCC AGATGGGGAA

      1150       1160       1170       1180       1190       1200
TGTGGTACAA GCACCAGCCC CTGGACCTGA TCAGAATGTA CTTCGGCGAG AAGATCGGCC

      1210       1220       1230       1240       1250       1260
TGTACTTCGC CTGGCTGGGC TGGTACACCG GCATGCTGAT CCCTGCCGCC GTCGTGGGCC

      1270       1280       1290       1300       1310       1320
TGTGCGTGTT CTTCTACGGC CTGGTCACCA TGAACGAGTC CCAGGTGTCC CAGGAAATCT

      1330       1340       1350       1360       1370       1380
GCAAGGCCAC CGAGGTGTTC ATGTGCCCCC TGTGCGACAA GAACTGCAGC CTGCAGAGGC

      1390       1400       1410       1420       1430       1440
TGAACGACAG CTGCATCTAC GCCAAAGTGA CCTACCTGTT CGACAACGGC GGCACCGTGT

      1450       1460       1470       1480       1490       1500
TCTTCGCCAT CTTCATGGCT ATCTGGGCTA CCGTGTTCCT GGAATTTTGG AAGAGAAGGC

      1510       1520       1530       1540       1550       1560
GGAGCATCCT GACCTACACC TGGGACCTGA TCGAGTGGGA GGAAGAGGAA GAGACACTGA

      1570       1580       1590       1600       1610       1620
GGCCCCAGTT CGAGGCCAAG TACTACAGAA TGGAAGTGAT CAACCCCATC ACCGGCAAGC

      1630       1640       1650       1660       1670       1680
CTGAGCCCCA CCAGCCCAGC AGCGACAAAG TGACCAGACT GCTGGTGTCC GTGTCCGGCA

      1690       1700       1710       1720       1730       1740
TCTTCTTCAT GATCAGCCTG GTCATCACCG CCGTGTTCGC CGTGGTGGTG TACAGACTGG

      1750       1760       1770       1780       1790       1800
TGGTCATGGA ACAGTTCGCC AGCTTCAAGT GGAACTTCGT GAAGCAGCAC TGGCAGTTCG

      1810       1820       1830       1840       1850       1860
CCACCAGCGG AGCCGCCGTG TGCATCAACT TTATCATCAT CATGCTGCTG AACCTGGCCT

      1870       1880       1890       1900       1910       1920
ATGAGAAGAT CGCCTACCTG CTGACCAACC TGGAATACCC CAGAACCGAG TCCGAGTGGG

      1930       1940       1950       1960       1970       1980
AGAACAGCTT CGCCCTGAAG ATGTTCCTGT TCCAGTTCGT GAACCTGAAC AGCTCTATCT

      1990       2000       2010       2020       2030       2040
TCTATATCGC CTTCTTCCTG GGCCGCTTCG TGGGCCACCC CGGCAAGTAC AACAAGCTGT

      2050       2060       2070       2080       2090       2100
TCGAGAGGTG GCGGCTGGAA GAGTGCCACC CCAGCGGCTG CCTGATCGAC CTGTGCCTGC

      2110       2120       2130       2140       2150       2160
AGATGGGCGT GATCATGTTC CTGAAGCAGA TTTGGAACAA CTTCATGGAA CTGGGCTACC
```

Fig. 7C

```
      2170       2180       2190       2200       2210       2220
CCCTGATCCA GAACTGGTGG TCCAGACACA AGATCAAGAG AGGCATCCAG GACGCCAGCA

      2230       2240       2250       2260       2270       2280
TCCCCCAGTG GGAGAATGAC TGGAACCTGC AGCCCATGAA CATCCACGGC CTGATGGACG

      2290       2300       2310       2320       2330       2340
AGTACCTGGA AATGGTGCTG CAGTTCGGCT TCACCACCAT CTTCGTGGCC GCTTTCCCCC

      2350       2360       2370       2380       2390       2400
TGGCCCCTCT GCTGGCCCTG CTGAACAACA TCATCGAGAT CAGACTGGAC GCCTACAAGT

      2410       2420       2430       2440       2450       2460
TCGTGACCCA GTGGCGGAGG CCCCTGCCTG CCAGAGCCAC AGACATCGGC ATCTGGCTGG

      2470       2480       2490       2500       2510       2520
GCATCCTGGA AGGCATCGGA ATCCTGGCCG TGATCACAAA CGCCTTCGTG ATCGCCATCA

      2530       2540       2550       2560       2570       2580
CCAGCGATTA CATCCCCCGC TTCGTGTACG AGTATAAGTA CGGCCCCTGC GCCAACCACG

      2590       2600       2610       2620       2630       2640
TGAAGCAGAA CGAGAACTGC CTGAAGGGCT ACGTGAACAA CAGCCTGAGC TTCTTCGACC

      2650       2660       2670       2680       2690       2700
TGTCCGAGCT GGGCATGGGC AAGAGCGGCT ACTGCAGATA CAGAGACTAC AGAGGCCCCC

      2710       2720       2730       2740       2750       2760
CTTGGAGCAG CAAGCCCTAC GAGTTCACCC TGCAGTACTG GCACATCCTG GCCGCCAGAC

      2770       2780       2790       2800       2810       2820
TGGCCTTCAT CATCGTGTTC GAGCACCTGG TGTTCGGCAT CAAGTCCTTC ATTGCCTACC

      2830       2840       2850       2860       2870       2880
TGATCCCCGA CATCCCCAAG GGCCTGAGAG AGAGAATCAG ACGCGAGAAG TACCTGGTGC

      2890       2900       2910       2920       2930       2940
AGGAAATGAT GTACGAGGCT GAGCTGGAAC ATCTGCAGCA GCAGAGAAGA AAGAGCGGCC

      2950       2960       2970       2980       2990       3000
AGCCCATCCA CCACGAGTGG CCTGAATTCT TAATTAA
```

Fig. 8A

```
        10         20         30         40         50         60
GGCGCGCCGG ATCCGCCACC ATGGAAGCCA GCAGCAGCGG CATCACCAAC GGCAAGAACA

        70         80         90        100        110        120
AGGTGTTCCA CGCCGAGGGC GGCCTGGACC TGCAGAGCCA CCAGCTGGAC ATGCAGATCC

       130        140        150        160        170        180
TGCCCGACGG CCCCAAGAGC GACGTGGACT TCAGCGAGAT CCTGAACGCC ATCCAGGAAA

       190        200        210        220        230        240
TGGCCAAGGA CGTCAACATC CTGTTCGACG AGCTGGAAGC CGTGAACAGC CCCTGCAAGG

       250        260        270        280        290        300
ACGACGACAG CCTGCTGCAC CCCGGCAACC TGACCAGCAC CAGCGAGGAC ACCAGCAGAC

       310        320        330        340        350        360
TGGAAGCTGG CGGCGAGACA GTGCGCGAGA GAAACAAGAG CAACGGCCTG TACTTCAGGG

       370        380        390        400        410        420
ACGGCAAGTG CAGAATCGAC TACATCCTGG TGTACAGAAA GAGCAACCCC CAGACCGAGA

       430        440        450        460        470        480
AGAGAGAGGT GTTCGAGAGG AACATCAGAG CCGAGGGCCT GCAGATGGAA AAAGAGAGCA

       490        500        510        520        530        540
GCCTGATCAA CAGCGACATC ATCTTCGTGA AGCTGCACGC CCCCTGGGAG GTGCTGGGCA

       550        560        570        580        590        600
GATACGCCGA GCAGATGAAC GTGCGGATGC CCTTCAGACG GAAAATCTAC TACCTGCCCA

       610        620        630        640        650        660
GGCGGTACAA GTTCATGAGC AGGATCGACA AGCAGATCAG CAGGTTCAGA CGGTGGCTGC

       670        680        690        700        710        720
CCAAGAAACC CATGAGACTG GACAAAGAGA CACTGCCCGA CCTGGAAGAG AACGACTGCT

       730        740        750        760        770        780
ACACCGCCCC CTTCAGCCAG CAGAGAATCC ACCACTTCAT CATCCACAAC AAGGACACAT

       790        800        810        820        830        840
TCTTCAACAA CGCCACCAGA TCCAGGATCG TGCACCACAT CCTGCAGAGG ATTAAGTACG

       850        860        870        880        890        900
AGGAAGGGAA GAACAAGATC GGCCTGAACA GACTGCTGAC CAACGGCAGC TACGAGGCCG

       910        920        930        940        950        960
CCTTCCCACT GCACGAGGGC AGCTACAGAA GCAAGAACAG CATCAAGACC CACGGCGCTG

       970        980        990       1000       1010       1020
TGAACCACAG ACATCTGCTG TACGAGTGCT GGGCCAGCTG GGGCGTGTGG TACAAGTACC

      1030       1040       1050       1060       1070       1080
AGCCCCTGGA CCTCGTGCGG AGATACTTCG GCGAGAAGAT CGGACTGTAC TTCGCCTGGC
```

Fig. 8B

```
      1090       1100       1110       1120       1130       1140
TGGGCTGGTA CACCGGCATG CTGTTCCCTG CCGCCTTTAT CGGCCTGTTC GTGTTCCTGT

      1150       1160       1170       1180       1190       1200
ACGGCGTGAC CACCCTGGAC CACTGCCAGG TGTCCAAAGA AGTGTGCCAG GCCACCGACA

      1210       1220       1230       1240       1250       1260
TCATCATGTG CCCCGTGTGC GACAAGTACT GCCCCTTCAT GAGACTGAGC GACAGCTGCG

      1270       1280       1290       1300       1310       1320
TGTACGCCAA AGTGACCCAC CTGTTCGACA ACGGCGCCAC CGTGTTCTTC GCCGTGTTCA

      1330       1340       1350       1360       1370       1380
TGGCCGTGTG GGCTACCGTG TTCCTGGAAT TTTGGAAGAG GCGGAGAGCC GTGATCGCCT

      1390       1400       1410       1420       1430       1440
ACGACTGGGA CCTGATCGAC TGGGAGGAAG AAGAGGAAGA GATCCGGCCC CAGTTCGAGG

      1450       1460       1470       1480       1490       1500
CCAAGTACAG CAAGAAAGAA CGGATGAACC CCATCAGCGG CAAGCCCGAG CCCTACCAGG

      1510       1520       1530       1540       1550       1560
CCTTCACCGA CAAGTGCAGC AGACTGATCG TGTCCGCCAG CGGCATCTTC TTCATGATCT

      1570       1580       1590       1600       1610       1620
GCGTCGTGAT CGCCGCCGTG TTCGGCATCG TGATCTACAG AGTGGTCACC GTGTCCACCT

      1630       1640       1650       1660       1670       1680
TCGCCGCCTT CAAGTGGGCC CTGATCAGAA ACAACAGCCA GGTGGCCACC ACCGGCACCG

      1690       1700       1710       1720       1730       1740
CCGTGTGTAT CAACTTCTGC ATCATCATGC TGCTGAACGT CCTGTACGAG AAGGTGGCCC

      1750       1760       1770       1780       1790       1800
TGCTGCTGAC AAACCTGGAA CAGCCCAGAA CCGAGAGCGA GTGGGAGAAC AGCTTCACCC

      1810       1820       1830       1840       1850       1860
TGAAGATGTT TCTGTTTCAG TTCGTGAACC TGAACAGCTC TACCTTCTAT ATCGCCTTCT

      1870       1880       1890       1900       1910       1920
TCCTGGGACG GTTCACCGGC CACCCTGGCG CCTACCTGAG ACTGATCAAC CGGTGGCGGC

      1930       1940       1950       1960       1970       1980
TGGAAGAGTG CCACCCCAGC GGCTGCCTGA TCGACCTGTG CATGCAGATG GGCATCATTA

      1990       2000       2010       2020       2030       2040
TGGTCCTGAA GCAGACCTGG AACAACTTCA TGGAACTGGG CTACCCCCTG ATCCAGAACT

      2050       2060       2070       2080       2090       2100
GGTGGACCAG ACGGAAAGTG CGGCAGGAAC ACGGCACCGA GAGAAAGATC AACTTCCCCC

      2110       2120       2130       2140       2150       2160
AGTGGGAGAA GGACTACAAC CTGCAGCCCA TGAACGCCTA CGGCCTGTTT GACGAGTACC
```

Fig. 8C

```
      2170       2180       2190       2200       2210       2220
TGGAAATGAT CCTGCAGTTC GGCTTCACCA CCATCTTCGT GGCCGCTTTC CCCCTGGCCC

      2230       2240       2250       2260       2270       2280
CCCTGCTGGC TCTGCTGAAC AACATCATCG AGATCAGACT GGACGCCTAC AAGTTCGTGA

      2290       2300       2310       2320       2330       2340
CCCAGTGGCG GAGGCCCCTG GCTAGCAGAG CCAAGGACAT CGGCATTTGG TACGGCATCC

      2350       2360       2370       2380       2390       2400
TGGAAGGCAT CGGCATCCTG AGCGTGATCA CCAACGCCTT CGTGATCGCT ATCACCAGCG

      2410       2420       2430       2440       2450       2460
ACTTCATCCC CAGACTGGTG TACGCCTATA AGTACGGCCC CTGTGCTGGC CAGGGCGAGG

      2470       2480       2490       2500       2510       2520
CTGGACAGAA ATGCATGGTC GGATACGTGA ACGCCAGCCT GAGCGTGTTC AGAATCAGCG

      2530       2540       2550       2560       2570       2580
ACTTCGAGAA CAGAAGCGAG CCCGAGAGCG ACGGCAGCGA GTTCAGCGGC ACCCCCCTGA

      2590       2600       2610       2620       2630       2640
AGTACTGCAG ATACAGAGAC TACAGGGACC CCCCCCACAG CCTGGCCCCT TACGGCTACA

      2650       2660       2670       2680       2690       2700
CCCTGCAGTT CTGGCACGTG CTGGCCGCCA GACTGGCCTT CATCATCGTG TTCGAGCACC

      2710       2720       2730       2740       2750       2760
TGGTGTTCTG CATCAAGCAC CTGATCAGCT ACCTGATCCC CGACCTGCCC AAGGACCTGA

      2770       2780       2790       2800       2810       2820
GAGACAGAAT GCGGAGAGAG AAGTACCTGA TTCAGGAAAT GATGTACGAG GCCGAGCTGG

      2830       2840       2850       2860       2870       2880
AAAGACTGCA GAAAGAGCGC AAAGAGCGGA AGAAGAACGG CAAGGCCCAC CACAACGAGT

      2890       2900       2910       2920       2930       2940
GGCCCGAATT CTTAATTAA
```

Fig. 9A

```
        10         20         30         40         50         60
GGCGCGCCGG ATCCGCCACC ATGGTCGAAC AGGAAGGCCT GACCGCCAAA GAGATCGACT

        70         80         90        100        110        120
ACGCCTTCCA GCAGAACGAG AACCTGGGCA GCAAAGAGAC AAGCTTCCTG ATCCCCGAGG

       130        140        150        160        170        180
ACCTGCAGAG CCCCCCTGAG AAGAGATTCA ACCTGTTCCT GAGAAGGCGG CTGATGTTCC

       190        200        210        220        230        240
AGAGAAGCGA GCACAGCAAG GACAGCGTGT TCTTCAGGGA CGGCATCAGA CAGATCGACT

       250        260        270        280        290        300
TCGTGCTGAG CTACGTCGAG GATCTGAAGA AGGACGGCGA GCTGAAGGCC GAGAGAAGAA

       310        320        330        340        350        360
GAGAGTTCGA GCAGAACCTG AGAAAGACCG GCCTGGACCT GGAAACCGAG GACAAGCTGA

       370        380        390        400        410        420
ACAGCGAGGA CGGCAAGACC TACTTCGTGA AGATCCACGC CCCCTGGGAG GTGCTGGTCA

       430        440        450        460        470        480
CATACGCTGA AGTGCTGGGC ATCAAGATGC CTATCAAGCT GAGCGACATC CCCAGACCCA

       490        500        510        520        530        540
AGTACCCCCC CCTGTCCTAC ATGCTGGGCG CCGTGAAGCT GCCCAGCAGC GTGAAGTACC

       550        560        570        580        590        600
CTACCCCCGA GTACTTCACC GCCCAGTTCA GCAGACACAG ACAGGAACTG TTTCTGATCG

       610        620        630        640        650        660
AGGACGAGGC CACATTCTTC CCAAGCAGCA CCAGAAACCG GATCGTGTAC TACATCCTGA

       670        680        690        700        710        720
GCAGATGCCC CTTCGGCGTG GAAGAGGGCA AGAAGAAGAT CGGCATCGAG AGACTGCTCA

       730        740        750        760        770        780
ACAGCAACAC CTACCTGAGC GCCTACCCCC TGCACGACGG ACAGTACTGG AAGCCCAGCA

       790        800        810        820        830        840
AGACCACCAG GCCCAACGAG AGGTACAACC TGTGCAAGAA CTGGGCCAGA TTCAGCTACT

       850        860        870        880        890        900
TCTACAAAGA GCAGCCCTTC CACCTGATCC GGAACTACTT CGGCGAAAAG ATCGGGATCT

       910        920        930        940        950        960
ACTTTGTGTT CCTGGGCTAC TACACCGAGA TGCTGCTGTT CGCCGCCCTC GTGGGACTGG

       970        980        990       1000       1010       1020
CCTGCTTCAT CTACGGCCTG CTGAGCATGG AAAACAACAG AACCAGCACC GAAATCTGCG

      1030       1040       1050       1060       1070       1080
ACCCCGACAT CGGCGGCCAG ATGATCATGT GCCCCCTGTG CGACGAAGTG TGCGACTACT
```

Fig. 9B

```
      1090       1100       1110       1120       1130       1140
GGCGGCTGAA CACCACCTGT CTGCACTCCA AGTTCAGCCA CCTGTTCGAT AACGAGAGCA

      1150       1160       1170       1180       1190       1200
CAGTGTTCTT CGCCCTGTTC ATGGGAATCT GGGTCACCCT GTTCCTCGAA TTTTGGAAGC

      1210       1220       1230       1240       1250       1260
AGAGACAGGC CAGACTGGAA TACGAGTGGG ACCTGGTGGA CTTCGAGGAA GAACAGCAGC

      1270       1280       1290       1300       1310       1320
AGCTGCAGCT CAGACCCGAG TTCGAGGCCA TGTGCAAGCA CAAGAAAATG AACCCCGTGA

      1330       1340       1350       1360       1370       1380
CCAAAGAAAT GGAACCCCAC ATGCCCCTGT GCCACAGAAT CCCTTGGTAC TTCGTGTCCG

      1390       1400       1410       1420       1430       1440
GCACCACCGT GACCTTCGGC ATGGCTCTGC TGCTGAGTAG CATGGTGTCC ATCCTGATCT

      1450       1460       1470       1480       1490       1500
ACAGACTGAG CGTGTTCGCC ACCTTCGCCA GCTTCATGGA AAGCGAGGCC ACCCTGCAGT

      1510       1520       1530       1540       1550       1560
CCGTGAAGAG TTTCTTCACA CCCCAGCTGG CCACCGCCCT GAGCGGCTCT TGCCTGAACT

      1570       1580       1590       1600       1610       1620
GCATCGTGAT CCTGATCCTC AACTTCTTCT ACGAGAAGAT CAGCGCCTGG ATCACCAAGA

      1630       1640       1650       1660       1670       1680
TGGAAATCCC TAGAACCCAC CAGGAATATG AGAGCAGCCT GACCCTGAAG ATGTTCCTGT

      1690       1700       1710       1720       1730       1740
TCCAGTTCGT GAACTACTAC AGCTCCTGCT TCTACGTGGC CTTCTTCAAG GGCAAGTTCG

      1750       1760       1770       1780       1790       1800
TGGGCTACCC CGGCAGCTAC ACCTACATGT TCAACATCTG GCGGAGCGAG GAATGCGGCC

      1810       1820       1830       1840       1850       1860
CTGCCGGCTG TCTGATCGAA CTGACCACCC AGCTGACCAT CATCATGATC GGCAAGCAGA

      1870       1880       1890       1900       1910       1920
TTTTCGGCAA CATCCACGAG GCTTTCCAGC CCCTGATCTT TAACTGGTGG CGCAGAAGAA

      1930       1940       1950       1960       1970       1980
GGGCCAGAAC CCACAGCGAG AAGCTGTACT CCAGATGGGA GCAGGACCAC GACCTCCAGG

      1990       2000       2010       2020       2030       2040
TGTACGGCCA CAGAGGCCTG TTCTACGAGT ATCTGGAAAC AGTGATCCAG TTCGGCTTCG

      2050       2060       2070       2080       2090       2100
CCACACTGTT CGTGGCTAGC TTCCCCCTGG CCCCTCTGTT CGCCCTGATG AACAACATCA

      2110       2120       2130       2140       2150       2160
TGGGCATCAG AGTGGACGCC TGGAAGCTGA CCACACAGTA CAGACGGCCC GTGGCCGCCA
```

Fig. 9C

```
      2170       2180       2190       2200       2210       2220
AGGCTCACTC TATTGGCGTG TGGCAGGACA TCCTGTTTGG CATGGCCATC GTGTCCGTGG

      2230       2240       2250       2260       2270       2280
CCACCAACGC CTTCATCGTG TCTTTCACCA GCGACATCAT CCCCAGGCTG GTGTACTTCT

      2290       2300       2310       2320       2330       2340
ACGCCTACAG CACCAACAGC ACCGAGCCCC TGTCCGGCTA CGTGAACAAC AGCCTGTCCG

      2350       2360       2370       2380       2390       2400
TGTTCCTGAT CGCTGACTTC CCCAACCACA CCGTGCCCAT GGAAAAGAAA GACTTCGTGA

      2410       2420       2430       2440       2450       2460
CCTGCCGGTA CAGGGACTAC AGATACCCCC CCGACCACGA GGATAAGTAC AGCCACAACA

      2470       2480       2490       2500       2510       2520
TGCAGTTTTG GCACGTGCTG GCCGCTAAGA TGACCTTCAT CATCGTGATG GAACACGTGG

      2530       2540       2550       2560       2570       2580
TGTTTCTGTT CAAGTTCCTG CTGGCCTGGC TGATCCCTGA CGTGCCCAAG GACGTGGTGG

      2590       2600       2610       2620       2630       2640
AAAAGATCAA GAGGGAAAAG CTGATGACCA TCAAGATCAT CCACGATTTC GAGCTGAACA

      2650       2660       2670       2680       2690       2700
AGCTCAAAGA GAATCTGGAC GTCGAGTACG GGAACATCAT GAAGAACGTG CTGGTGGACG

      2710       2720       2730       2740       2750       2760
AGGACAACTC CCTGAAGGCC AAGACCACAG TGGAATTCTT AATTAA
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METHOD FOR SCREENING A MODULATOR OF A TMEM16 FAMILY MEMBER

This application claims priority to and the benefit of the U.S. Provisional Application No. 61/624,491, filed on Apr. 16, 2012, the entire content of which is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to a method for screening a modulator of a TMEM16 family member.

BACKGROUND ART

Phospholipids and glycosphingolipids are distributed asymmetrically in plasma membrane leaflets, with phosphatidylserine (PS) and phosphatidylethanolamine (PE) in the inner leaflet, and phosphatidylcholine (PC), galactosylceramide (GalCer) and glucosylceramide (GluCer) mainly in the outer leaflet (1,2). The lipid asymmetry is disrupted in various processes, including apoptotic cell death (3), activated platelets (4), red blood cell aging (5), pyrenocyte formation in definitive erythropoiesis (6), fusion of macrophages, myocytes, or cytotrophoblasts (7-9), and sperm capacitation (10).

Distribution of lipids in plasma membranes is regulated by three types of lipid transporters: flippases, floppases and scramblases. Flippases, also called ATP-dependent aminophospholipid translocases, transport aminophospholipids from the extracellular leaflet to the cytoplasmic side (1,11). The type IV-P-type ATPases (P4-ATPase), a subfamily of the P-type ATPase multispan transmembrane proteins, are strong candidates for flippases (12). Floppases are transporters that move a wide range of lipids from the cytosolic to the extracellular leaflet in an ATP-dependent manner. The ATP-binding cassette (ABC) ATPase, particularly ABCA1, has been proposed as a floppase (13), but ABCA1-deficient cells exhibit no defects in transbilayer phospholipid movement (14) arguing against this role.

Once established, the phospholipid distribution between the outer and inner leaflets is not easily disrupted; ATP-dependent translocase inactivation alone does not appear sufficient to cause the rapid PS exposure seen in apoptotic cell death and platelet activation. Thus, a phospholipid scramblase that bi-directionally and non-specifically transports phospholipids in response to Ca2+ has been proposed (15). Using a liposome reconstitution system with synthetic phospholipids, Basse et al. (16) purified a 37-kDa protein from human erythrocytes, and named it phospholipid scramblase (PLSCR). Its cDNA was then isolated (17). However, since the Ca^{2+}-induced PS exposure is normal in PLSCR1$^{-/-}$ cells (18), PLSCR's function as a phospholipid scramblase has been challenged (15,19).

By repeatedly selecting cell populations that efficiently exposed PS in response to Ca^{2+} ionophore, we recently established a subline of mouse pro B cell line (Ba/F3) that constitutively exposes PS (20). The Ba/F3 subline harbours a mutated form of TMEM16F protein, a protein carrying eight transmembrane regions with cytoplasmic N- and C-termini. Ba/F3 cells carrying the mutated form of TMEM16F constitutively exposed PS and PE, and internalized PC and SM. We thus proposed TMEM16F as a phospholipid scramblase (20). Confirming that TMEM16F is a Ca2+-dependent phospholipid scramblase, recessive TMEM16F mutations were identified in human patients with Scott syndrome (20,21), which is known to result from a phospholipid-scrambling defect; these patients suffer from impaired blood clotting. However, it is not clear if TMEM16F is involved in other processes, such as apoptotic cell death or cell fusion. Two of the TMEM16 family's 10 members, TMEM16A and 16B, are Ca^{2+}-dependent Cl^- channels (22-24); this raises a question of whether TMEM16F is likewise a Cl^- channel, and whether any other TMEM16 family members are phospholipid scramblases.

SUMMARY OF INVENTION

We established an immortalized fetal thymocyte (IFET) cell line from fetal thymus of mice carrying a floxed TMEM16F allele. IFETs express TMEM16F, 16H, and 16K, and expose PS in response to a Ca^{2+} ionophore. Deleting TMEM16F in the IFETs completely abolished their ability to expose PS in response to Ca^{2+}-ionophore. On the other hand, Fas ligand (FasL) treatment efficiently induced PS exposure in the TMEM16F− deficient cells. In the presence of TMEM16C, 16D, 16F, 16G, and 16J, TMEM16F$^{-/-}$ IFETs responded to Ca^{2+} ionophore by scrambling phospholipids and galactosylceramide, while other family members did not. On the other hands, the two family members, TMEM16A and 16B, but not others showed the Ca^{2+}-dependent Cl^- channel activity. Based on those results, the present invention is archived.

The present invention provides:

1. A method for screening a modulator of a TMEM16 family member, which comprises the following steps:
(1) treating cells expressing the TMEM16 family member with a candidate of the modulator, and
(2) determining whether the candidate alters distribution of a lipid selected from phosphatidylserine, phosphatidylcholine, and galactosylceramide in plasma membrane of the cells,
wherein a candidate which increases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member, and
a candidate which increases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member;
2. The method of 1 above, wherein the TMEM16 family member is TMEM 16C and the lipid is selected from phosphatidylcholine and galactosylceramide;
3. The method of 1 above, wherein the TMEM16 family member is TMEM 16D and the lipid is selected from phosphatidylserine, phosphatidylcholine, and galactosylceramide;
4. The method of 1 above, wherein the TMEM16 family member is TMEM 16G and the lipid is selected from phosphatidylserine, phosphatidylcholine, and galactosylceramide; and
5. The method of 1 above, wherein the TMEM16 family member is TMEM 16J and the lipid is selected from phosphatidylserine, phosphatidylcholine, and galactosylceramide.

In another embodiment, the present invention provides the followings:

6. A method for screening a modulator of a TMEM16 family member, which comprises the following steps:

(1) treating cells expressing the TMEM16 family member with a candidate of the modulator, and

(2) determining whether the candidate alters distribution of a lipid selected from phosphatidylserine and phosphatidylcholine in plasma membrane of the cells,

wherein a candidate which increases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member, and

a candidate which increases distribution of phosphatidylcholine in the inner leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylcholine in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member;

7. The method of 6 above, wherein the TMEM16 family member is TMEM 16C and the lipid is phosphatidylcholine;

8. The method of 6 above, wherein the TMEM16 family member is TMEM 16D and the lipid is selected from phosphatidylserine and phosphatidylcholine;

9. The method of 6 above, wherein the TMEM16 family member is TMEM 16G and the lipid is phosphatidylcholine; and

10. The method of 6 above, wherein the TMEM16 family member is TMEM 16J and the lipid is phosphatidylcholine.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1**A-**1**D: Establishment of TMEM16F$^{-/-}$ IFET Cell Line.

A, Schematic representation of wild-type and mutant TMEM16F alleles together with the targeting vector. Recognition sites for Eco RI (E), Eco RV (V), Kpn I (K), and Sma I (S) in the flanking region of exon 2 (filled box) are indicated. In the target vector, a 1.0-kb DNA fragment carrying exon 2 and its flanking region was replaced by a 2.7-kb fragment carrying two loxP sequences (filled arrowhead) and PGK-neo (Neo^R) flanked by FRT sequences (gray arrowhead). Diphtheria toxin A-fragment (DT-A) driven by the tk promoter was inserted at 5' site of the vector. In NeoFRT allele, TMEM16F chromosomal gene was replaced by the targeting vector. In Floxed allele, the FRT-flanked NeoR gene was removed by FLPe recombinase. In deleted allele, the loxP-flanked exon 2 of TMEM16F gene was deleted by Cre recombinase. Primers used in FIG. **1**C are indicated by arrows. Scale bar, 1.0 kb.

B, Real-time PCR analysis for mRNA of TMEM16F family members in IFETs. An IFET cell line was established from TMEM16F$^{flox/flox}$ fetal thymocytes. TMEM16A-16H, 16J and 16K mRNA in TMEM16F$^{flox/flox}$ IFETs was quantified by real-time PCR, and expressed relative to β-actin mRNA. The experiment was carried out for three times, and the average value was plotted with S.D. (bar).

C, Deletion of TMEM16F exon 2 in the IFET cell line. TMEM16F$^{flox/flox}$ IFETs were infected by Cre-bearing adenovirus to establish TMEM16F$^{-/-}$ IFET cells. Chromosomal DNA from TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs was analyzed by PCR with the primers indicated in FIG. **1**A.

D, Western blots for TMEM16F in TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs. Cell lysates (10 μg proteins) were separated by 7.5% SDS-PAGE, and blotted with rabbit anti-TMEM16F serum (upper panel) or anti-α-tubulin antibody (lower panel). Molecular weight standards (Precision Plus Standard, Bio-Rad) are shown in kDa at left.

FIG. **2**A-**2**G: An Indispensable Role of TMEM16F for Ca^{2+}-Induced but not Apoptotic PS Exposure.

A, Ca^{2+} ionophore induced PS exposure. TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs were treated at 20° C. with 3.0 μM A23187 in the presence of Cy5-labeled Annexin V. Annexin V-binding to the cells was monitored by flow cytometry for 10 min, and expressed in MFI (mean fluorescence intensity).

B and C, Ca^{2+} ionophore induced lipid internalization. TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs were treated at 15° C. with 250 nM A23187 in the presence of 100 nM NBD-PC (B) or 250 nM NBD-GalCer (C). Using aliquots of the reaction mixture, the BSA-non extractable level of NBD-PC or NBD-GalCer in the SytoxBlue-negative population was determined at the indicated time by FACSAria, and expressed in MFI.

D, Transformation of IFETs with mouse Fas. TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs were infected with a retrovirus carrying mouse Fas, and were stained with a PE-labeled hamster mAb against mouse Fas. The staining profile of parental cells is also shown.

E-G, FasL-induced apoptosis. Fas-expressing TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs were treated at 37° C. for 2 h with 60 units/ml FasL in the absence or presence of 50 μM Q-VD-OPh. In E, the cells were permeabilized with 90% methanol, and stained with rabbit anti-active caspase 3 followed by incubation with Alexa 488-labeled goat anti-rabbit IgG. In F, cells were stained with Cy5-labeled Annexin V and PI and analyzed by FACSAria. In G, cells were analyzed by FACSAria before and after FasL treatment; the FSC and SSC profiles are shown.

FIG. **3**A-**3**B: Ca^{2+}-Dependent PS Exposure by TMEM16 Family Members.

The ten TMEM16 family members were FLAG-tagged at C-terminus and introduced into TMEM16F$^{-/-}$ IFETs to establish stable transformants.

A, Western blotting. TMEM16 protein expression in each transformant was analyzed by Western blotting with an anti-FLAG mAb. Note that the amount of TMEM16K lysate protein analyzed was one-eighth that of the others.

B, Ca^{2+}-induced PS exposure by TMEM16 family members. TMEM16F$^{-/-}$ IFETs transformed with the indicated TMEM16 family member were stimulated with 3.0 μM A23187. Annexin V binding was monitored with a FACSAria at 20° C. for 2 min, and expressed in MFI. The experiments were carried out for three times, and the average values were plotted with S.D. (bars).

FIG. **4**A-**4**C: Ca^{2+}-Dependent Internalization of NBD-PC and NBD-GalCer by TMEM16 Family Members.

A and C, The ability of TMEM16 family members to internalize NBD-PC and NBD-GalCer. TMEM16F$^{-/-}$ IFETs transformed with the indicated TMEM16 family member were treated at 15° C. with (+) or without (−) 250 nM A23187 in the presence of 100 nM NBD-PC for 4 min (A) or 250 nM NBD-GalCer for 5 min (C), and the internalized, or BSA-non extractable NBD-PC or NBD-GelCer, was quantified by FACSAria, and expressed in MFI.

B, Requirement of Ca^{2+} for the constitutive internalization of NBD-PC by TMEM16D. The TMEM16D transformants of $TMEM16F^{-/-}$ IFETs were treated with 40 μM BAPTA-AM for 30 min in Ca^{2+}-free RPMI, and incubated at 15° C. for 8 min in HBSS containing 1 mM $CaCl_2$ and 100 nM NBD-PC. The internalized NBD-PC was determined as above, and expressed as percentage of the internalized NBD-PC obtained without BAPTA-AM treatment.

All experiments in FIGS. **4**A, **4**B, and **4**C were carried out for three times, and the average values were plotted with S.D. (bars).

FIG. **5**A-**5**C: Ca^{2+}-Dependent Cl^--Channel Activity of TMEM16 Family Members.

A, Expression of TMEM16 family members in HEK293T cells. HEK293T cells were transfected with a pEF-BOS-EX vector carrying cDNA for the flag-tagged TMEM16 family member. Two days later, the expression level of each TMEM16 member was analyzed by Western blotting with anti-Flag and anti-α-tubulin mAbs. Note that the amount of TMEM16K lysate protein analyzed was one-eighth that of the others.

B, Ca^{2+} ionophore-induced TMEM16A and 16B Cl^--channel activity. HEK293T cells were co-transfected with a pEF-BOS-EX vector carrying TMEM16A or 16B cDNA, and pMAX-EGFP. Two days later, the Cl^--channel activity of EGFP-positive cells was examined by electrophysiology. The pipette (intracellular) solution contained 500 nM free Ca^{2+}. Representative whole-cell membrane currents elicited at −120 to +120 mV in 10 mV-steps are shown for vector-, TMEM16A-, and 16B-transfected cells. The holding membrane potential was maintained at 0 mV.

C, Outward rectification of the Cl^- current by TMEM16 family members. HEK293T cells were co-transfected with pMAX-EGFP and pEF-BOS-EX vector for the indicated TMEM16 family member, and electrophysiology was carried out as described above. Membrane currents were measured at the indicated voltage pulses (Vm). Experiments were independently done 3-5 times, and the average values were plotted against the applied membrane potential with S.D. (bars).

FIG. **6**A-**6**C: Real-Time PCR Analysis for TMEM16 Family Member mRNA in Mouse Tissues.

RNA was prepared from the indicated mouse tissues, and mRNA level quantified by real-time PCR were expressed relative to β-actin mRNA for each TMEM16 family member.

FIG. **7**A-**7**C: The Designed Nucleotide Sequence for Mouse TMEM16C.

The first 20 nucleotides carry Bam H1 (GGATCC) and Kozak sequence for ribosome-binding (CCACC) in front of ATG initiation codon. The coding sequence is followed by an Eco R1 recognition sequence (GAATTC).

FIG. **8**A-**8**C: The Designed Nucleotide Sequence for Mouse TMEM16D.

The first 20 nucleotides carry Bam H1 (GGATCC) and Kozak sequence for ribosome-binding (CCACC) in front of ATG initiation codon. The coding sequence is followed by an Eco R1 recognition sequence (GAATTC).

FIG. **9**A-**9**C: The Designed Nucleotide Sequence for Mouse TMEM16E.

The first 20 nucleotides carry Bam H1 (GGATCC) and Kozak sequence for ribosome-binding (CCACC) in front of ATG initiation codon. The coding sequence is followed by an Eco R1 recognition sequence (GAATTC).

DESCRIPTION OF EMBODIMENTS

"A TMEM16 family member" is a protein which has 8 transmembrane regions with cytosolic N- and C-termini. Although ten TMEM16 family members are known (35, 36), "a TMEM16 family member" in the present invention is selected from TMEM16C, TMEM16D, TMEM16G, and TMEM16J. The TMEM16 family member of the present invention may be derived from, but not limited to, human, monkey, mice, or rabbit. The amino acid sequences of human TMEM16C, TMEM16D, TMEM16G, and TMEM16J are disclosed under the NCBI reference number NP_113606.2 (TMEM16C), NP_849148.2 (TMEM16D), NP_001001666.1 (TMEM16G), and NP_001012302.2 (TMEM16J).

"A candidate of a modulator of a TMEM16 family member may be a natural or synthetic product, and may be low-molecular compounds, proteins, nucleic acid molecules, peptides, antibodies, or cell extract or culture supernatant of microorganisms, plants or animals. The candidate may be provided in a form of a library, such as a library of low-molecular compounds, peptides, or antibodies.

As used herein, "cells expressing a TMEM16 family member" includes cells which express the TMEM16 family member in nature from the genome, and cells which express the TMEM16 family member from a gene encoding the TMEM16 family member which has been introduced into the cells. The cells may be derived from, but not limited to, human, monkey, mice, or rabbit. For example, human HeLa, human EBV (Epstein Barr Virus)-transformed B cell line, mouse MEF (embryonal fibroblasts), and mouse pro B cell line Ba/F3 may be used in the present invention.

"A modulator of a TMEM16 family member" includes both "a modulator enhancing a function of a TMEM16 family member" and "a modulator suppressing a function of a TMEM16 family member". As used herein, "enhancing (or suppressing) a function of a TMEM16 family member" means promoting (or inhibiting) a biological function of a TMEM16 family member as a lipid scramblase in cells or animals. "A modulator of a TMEM16 family member" may be an agent directly affecting the function of the TMEM16 family member protein, or an agent increasing or decreasing expression of the TMEM16 family member. "Increasing or decreasing expression of a TMEM16 family member" includes increasing or decreasing mRNA expression from a gene encoding the TMEM16 family member, and increasing or decreasing protein expression of the TMEM16 family member. Therefore, "a modulator of a TMEM16 family member" includes an agent affecting a regulatory sequence of a gene encoding the TMEM16 family member such as a promoter or enhancer, and also includes an antisense oligonucleotide (DNA or RNA), siRNA, miRNA, and lybozyme prepared according to the sequence of the gene encoding the TMEM16 family member.

In the method of the present invention, the enzymatic activity of a TMEM16 family member as a lipid scramblase is measured. The lipid is selected from the group consisting of phosphatidylserine (PS), phosphatidylcholine (PC), and galactosylceramide (GalCer). Under the normal condition, PS is distributed in the inner leaflet of plasma membrane and PC and GalCer are distributed in the outer leaflet of plasma membrane. The TMEM16 family member of the present invention moves PS to the outer leaflet of plasma membrane (i.e., exposes PS) and moves PC and GalCer to the inner leaflet of plasma membrane (i.e., internalizes PC and GalCer). The enzymatic activity of a TMEM16 family member may be measured by determining distribution of the lipid in plasma membrane.

A candidate which increases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylserine in the outer

leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member. A candidate which increases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of the TMEM16 family member, and a candidate which decreases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of the TMEM16 family member. As used herein, "control" means distribution of the same lipid in the same leaflet in cells expressing the same TMEM16 family member in the absence of the candidate of the modulator.

TMEM16C functions as a scramblase for PC and GalCer. Accordingly, a candidate which increases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator which enhancing a function of TMEM16C, and a candidate which decreases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16C.

TMEM16D functions as a scramblase for PS, PC, and GalCer. Accordingly, a candidate which increases distribution of PS in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of TMEM16D, and a candidate which decreases distribution of PS in the outer leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16D. Also, a candidate which increases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator which enhancing a function of TMEM16D, and a candidate which decreases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16D.

TMEM16G functions as a scramblase for PS, PC, and GalCer. Accordingly, a candidate which increases distribution of PS in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of TMEM16G, and a candidate which decreases distribution of PS in the outer leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16G. Also, a candidate which increases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator which enhancing a function of TMEM16G, and a candidate which decreases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16G.

TMEM16J functions as a scramblase for PS, PC, and GalCer. Accordingly, a candidate which increases distribution of PS in the outer leaflet of plasma membrane compared to control is selected as a modulator enhancing a function of TMEM16J, and a candidate which decreases distribution of PS in the outer leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16J. Also, a candidate which increases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator which enhancing a function of TMEM16J, and a candidate which decreases distribution of PC or GalCer in the inner leaflet of plasma membrane compared to control is selected as a modulator suppressing a function of TMEM16J.

In the step "(1) treating cells expressing the TMEM16 family member with a candidate of the modulator", typically, the candidate is added to the culture medium of the cells in the presence of Ca^{2+}. When appropriate, calcium ionophore such as A23187 may be added to the culture medium at the same time or after the addition of the candidate.

Distribution of PS in plasma membrane may be determined by detecting the binding between PS exposed to the cell surface and an agent which has a property to bind to PS, such as AnnexinV or MFG-E8 (also called as lactadherin). For example, cells expressing a TMEM16 family member which has been treated with a candidate are treated with fluorescently-labelled AnnexinV and the amount of AnnexinV bound to the cell surface is measured.

Distribution of PS in plasma membrane also may be determined based on blood-clotting reaction. For example, cells expressing a TMEM16 family member are treated with a candidate of the modulator and also with calcium ionophore concurrently with or after the treatment with the candidate, and mixed with agents required for blood coagulation such as factor Xa, factor Va, and prothrombin, and then production of thrombin is measured. Alternatively, fibrinogen may be further added to the cell culture to measure production of fibrin.

Distribution of PC and GalCer in plasma membrane may be determined by using a fluorescently-labeled lipid. As a fluorescent label, NBD and TopFluor may be used. For example, a fluorescently-labeled lipid is added to the culture medium such that the fluorescently-labeled lipid is incorporated into outer leaflet of plasma membrane of cells expressing a TMEM16 family member. When the TMEM16 family member functions as a lipid scramblase, the fluorescently-labeled lipid is moved to the inner leaflet of plasma membrane (i.e., internalized). Therefore, cells expressing a TMEM16 family member may be treated with a candidate of the modulator, and also with calcium ionophore if needed, in the presence of a fluorescently-labeled lipid such as NBD-PC or NBD-GalCer. The cells were then treated with BSA to remove unincorporated NBD-PC or NBD-GalCer, followed by measuring the NBD-PC or NBD-GalCer incorporated into cell by a flow cytometry.

Abnormality (mutations and over-expression) in TMEM16 family members is known to cause various human diseases (36). For example, genetic mutations in TMEM16C, 16E, 16F and 16K are associated with craniocervical dystonia (58), musculoskeletal disorder (49, 51), bleeding disorder (20), and ataxia (52), respectively. TMEM16A and 16G are over-expressed in human gastrointestinal stromal tumors/head and neck squamous carcinoma, and prostate cancer, respectively (59, 60). Therefore, the method of the present invention is useful for the development of therapeutic or prophylactic agents for such diseases.

Example

Experimental Procedures

Materials and Cell Lines

Leucine-zipper-tagged human FasL was produced in COS7 cells as described (25). One unit of FasL is defined as the activity that kills 1.0×10^5 mouse WR19L cell expressing Fas (W3 cells) in 4 h. A caspase inhibitor, Q-VD-OPh (quinolyl-valyl-O— methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone) was purchased from R&D systems (Minneapolis, Minn.). IFETs were maintained in RPMI medium containing 10% FCS (Nichirei Bioscience, Tokyo, Japan) and 50 μM β-mercaptoethanol. HEK293T and Plat-E cells (26) were cultured in DMEM containing 10% FCS.

cDNA Cloning

Mouse TMEM16F cDNA (NCBI: NM_175344) was described (20). Mouse cDNAs for TMEM16A (GenBank: BC062959.1), 16B (GenBank: BC033409.1), and 16G (GenBank: BC116706.1) were from DNAFORM (Yokohama, Japan). Mouse cDNAs for TMEM16C (NCBI: NM_001128103.1), 16D (Ensemble: ENSMUST00000070175), and 16K (NCBI: NM_133979.2) were cloned from brain tissue by RT-PCR, while cDNAs for TMEM16E (NCBI: NM_177694.5), 16H (NCBI: NM_001164679.1), and 16J (NCBI: NM_178381.3) were isolated from the skeletal muscle, thymus, and stomach, respectively. All cDNAs were verified by sequencing. The following primers were used to isolate TMEM16 cDNAs (the extra sequence for the restriction enzyme is underlined):

(SEQ ID NO: 1)
TMEM16A, 5'-ATATGGATCCACCATGAGGGTCCCCGAGAAGTA,
and
(SEQ ID NO: 2)
5'-ATATGAATTCCAGCGCGTCCCCATGGTACT;

TMEM16B,
(SEQ ID NO: 3)
5'-ATATGAATTCCGCATGCACTTTCACGACAACCA,
and
(SEQ ID NO: 4)
5'-ATATGAATTCTACATTGGTGTGCTGGGACC;

TMEM16C,
(SEQ ID NO: 5)
5'-ATATGGATCCAAAATGGTCCACCACTCAGGCTC,
and
(SEQ ID NO: 6)
5'-ATATCAATTGAGGCCATTCATGGTGAATAG;

TMEM16D,
(SEQ ID NO: 7)
5'-ATATAGATCTAAAATGGAGGCCAGCTCTTCTGG,
and
(SEQ ID NO: 8)
5'-ATATCAATTGTGGCCACTCATTGTGATGTG;

TMEM16E,
(SEQ ID NO: 9)
5'-ATATGGATCCGAGATGGTGGAGCAGGAAGGCTT,
and
(SEQ ID NO: 10)
5'-ATATCAATTGGACTGTAGTTTTAGCCTTCA;

TMEM16G,
(SEQ ID NO: 11)
5'-ATATAGATCTGACATGCTGCGGGGGCAAGCGCG,
and
(SEQ ID NO: 12)
5'-ATATGAATTCGCCTCCGGTAACCCCTACTG;

TMEM16H,
(SEQ ID NO: 13)
5'-ATATAGATCTGCCATGGCCGAGGCGGCTTCGGG,
and
(SEQ ID NO: 14)
5'-ATATGAATTCAGGCCTGTGACCTGCGTCCT;

TMEM16J,
(SEQ ID NO: 15)
5'-ATATGAATTCAGCATGCAGGATGATGAGAGTTC,
and
(SEQ ID NO: 16)
5'-ATATCAATTGTACATCCGTGCTCCTGGAAC;

TMEM16K,
(SEQ ID NO: 17)
5'-ATATGGATCCAAGATGAGAGTGACTTTATCAAC,
and
(SEQ ID NO: 18)
5'-ATATCAATTGGGTAGCTTCCTTCCCATCTT.

Since the native mouse cDNAs for TMEM16C, 16D, and 16E produced a low level of proteins in mammalian cells, sequences with enhanced mRNA stability and translational efficiency were custom ordered from GENEART (Regensburg, Germany) (FIGS. **7-9**, SEQ ID NOS: 19-21).

Establishment of TMEM16F$^{-/-}$ IFET Cell Line

TMEM16F conditionally targeted mice were generated by UNITECH (Chiba, Japan) as a custom order. In brief, a neo-loxP cassette carrying the PGK promoter-driven neo gene and flanked by FRT sequences was inserted into intron 3 of the TMEM16F gene (FIG. **1**A). A 1.0 kb-DNA fragment containing exon 2 was replaced with a fragment carrying the corresponding sequence and a loxP sequence. The diphtheria toxin A-fragment (DT-A) driven by the thymidine kinase (tk) promoter, was inserted at 5' end of the vector. Mouse Bruce-4 ES cells were transfected with the targeting vector by electroporation, and G418-resistant clones were screened for homologous recombination by PCR. Positive clones were injected into blastocysts to generate TMEM16F$^{+/NeoFRT}$ mice.

The TMEM16F$^{+/NeoFRT}$ mice were crossed with CAG-FLPe transgenic mice to remove the Neo cassette (27). Offspring were backcrossed to wild-type C57BL/6 mice to remove the CAG-FLPe transgene, generating TMEM16F$^{+/flox}$ mice. Mice were housed in a specific pathogen-free facility at Kyoto University, and all animal experiments were carried out in accordance with protocols approved by Kyoto University.

IFET cell lines were established as described (28). In brief, TMEM16F$^{+/flox}$ mice were intercrossed, and fetal thymocytes were obtained at embryonic day 14.5. Thymocytes were cultured in DMEM containing 10% FCS, 1× non-essential amino acids, 10 mM Hepes-NaOH buffer (pH 7.4), and 50 μM β-mercaptoethanol. Retroviruses carrying genes for H-ras^{V12} and c-myc were produced in Plat-E cells with pCX4 vector (29), concentrated by centrifugation, and attached to RetroNectin-coated plates (Takara Bio, Kyoto, Japan). Thymocytes were attached to the retrovirus-coated plate by centrifugation at 400×g for 5 min, and cultured in medium containing 5 ng/ml mouse IL-7 (PeproTech, Rocky Hill, N.J.) (30). The resultant IFETs were infected with 1×10^5 pfu/ml Adeno-Cre (Takara Bio) and cloned by limited dilution. Clones carrying the TMEM16F$^{-/-}$ allele were selected by PCR with following primers: wild-type specific sense primer, CTCCAGAGTTTGTAAGTAACACAT (SEQ ID NO: 22), mutant specific sense primer, CAGTCATCGATGAATTCATAACTT (SEQ ID NO: 23), and common anti-sense primer, AAGACTGATTTCCAAGG TTATCGAA (SEQ ID NO: 24).

Transformation of TMEM16F$^{-/-}$ IFETs

Mouse TMEM16 cDNAs were inserted into pMXs puro c-FLAG (20) to express proteins tagged with FLAG at the C-terminus. Retrovirus was produced in Plat-E cells, and used to infect TMEM16F$^{-/-}$ IFETs. Stable transformants were selected in medium containing 2 μg/ml puromycin. Mouse Fas cDNA (GenBank: NM_007987) was introduced into IFETs by retrovirus-mediated transformation, and its expression was confirmed by flow cytometry with an anti-Fas mAb (Jo2) (MBL, Nagoya, Japan).

Real-Time PCR

Total RNA was reverse-transcribed using Superscript III reverse-transcriptase (Invitrogen, Carlsbad, Calif.) or a High Capacity RNA-to-cDNA™ kit (Applied Biosystems, Foster City, Calif.). Aliquots of the products were amplified in a reaction mixture containing LightCycler®480 SYBR Green I Master (Roche Diagnostics, Basel, Switzerland). Primers used for real-time PCR were as follows: TMEM16A, 5'-AC-

CCCGACGCCGAATGCAAG (SEQ ID NO: 25), and 5'-GCTGGTCCTGCCTGACGCTG (SEQ ID NO: 26); 16B, 5'-GAGGCGCACACCTGGGTCAC (SEQ ID NO: 27), and 5'-ATGGGGCGTGGATCCGGACA (SEQ ID NO: 28); 16C, 5'-GCCAGCAATTGCCAACCCCG (SEQ ID NO: 29), and 5'-GCAGTCCGACTCCTCCAGCTCT (SEQ ID NO: 30); 16D, 5'-ACAGGCATGCTCTTCCCCGC (SEQ ID NO: 31), and 5'-GCGATCACTGCTCGGCGTCT (SEQ ID NO: 32); 16E, 5'-AGCAGCTCCAGCTTCGGCCT (SEQ ID NO: 33), and 5'-TTCACGCTCTGCAGGGTGGC (SEQ ID NO: 34); 16F, 5'-CCCACCTTTGGATCACTGGA (SEQ ID NO: 35), and 5'-TCGTATGCTTGTCTTTTCCT (SEQ ID NO: 36); 16G, 5'-ACATGTGCCCGCTGTGCTCC (SEQ ID NO: 37), and 5'-GGGCCGAGGCCTCTCCTCAA (SEQ ID NO: 38); 16H, 5'-TGGAGGAGCCACGTC-CCCAG (SEQ ID NO: 39), and 5'-GCGGGGCAGACCCT-TCACAC (SEQ ID NO: 40); 16J, 5'-GCTGTGGTGGT-GACTGGGGC (SEQ ID NO: 41), and 5'-CCAGGCGCGTGGATTTCCCA (SEQ ID NO: 42); 16K, 5'-TGGGGGCAGAAGCAGTCGGT (SEQ ID NO: 43), and 5'-GGCCTGTGGGTAGCCAGGGAT (SEQ ID NO: 44); β-actin, 5'-TGTGATGGTGGGAATGGGTCAG (SEQ ID NO: 45) and 5'-TTTGATGTCACGCAC-GATTTCC (SEQ ID NO: 46).

The mRNA was quantified at the point where Light Cycler System detected the upstroke of the exponential phase of PCR accumulation with the respective linearized plasmid DNA as reference.

Western Blotting

Cells were lysed in RIPA buffer [50 mM Hepes-NaOH buffer (pH 8.0) containing 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and protease inhibitor cocktail (cOmplete Mini, Roche Diagnostics)]. After removing debris, cell lysates were mixed with 5×SDS sample buffer [200 mM Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.05% Bromophenolblue], incubated at room temperature for 30 min, and separated by 10% SDS-PAGE (Bio Craft, Tokyo, Japan). After transferring proteins to a PVDF membrane (Millipore, Billerica, Mass.), membranes were probed with HRP-conjugated mouse anti-FLAG M2 (Sigma-Aldrich, St. Louis, Mo.), and peroxidase activity was detected using a Western Lightning®-ECL system (PerkinElmer, Waltham, Mass.).

To prepare rabbit antibody against mouse TMEM16F, the N-terminal region of mouse TMEM16F (amino acids from 1-289) was fused to glutathione-S-transferase (GST) in a pGEX-5X-1 vector (GE Healthcare, Buckinghamshire, England). The recombinant protein was produced in *E. coli*, purified with Glutathione-Sepharose, and used to immunize rabbits at Takara Bio as a custom order. Western blotting with the rabbit anti-TMEM16F and HRP-labeled goat anti-rabbit Ig (Dako, Copenhagen, Denmark) was carried out as described above using Immunoreaction Enhancer Solution (Can Get Signal®, Toyobo Life Science, Tokyo, Japan).

Analysis of PS Exposure

The Ca^{2+}-induced PS exposure were examined as described (20). In brief, 5×10^5 cells were stimulated at 20° C. with 3.0 μM A23187 in 500 μl of 10 mM Hepes-NaOH buffer (pH 7.4) containing 140 mM NaCl, 2.5 mM $CaCl_2$ and 5 μg/ml Propidium Iodide (PI), and 1000-fold-diluted Cy5-labeled Annexin V (Bio Vision, Milpitas, Calif.), and applied to the injection chamber of a FACSAria (BD Bioscience, Franklin Lakes, N.J.) set at 20° C.

Internalization of NBD-PC and NBD-GalCer

Cells (10^6) were stimulated at 15° C. with 250 nM A23187 in 1 ml Hank's Balanced Salt Solution (HBSS) (Gibco, Billings, Mont.) containing 1 mM $CaCl_2$, with a fluorescent probe, 100 nM 1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD-PC) (Avanti Polar Lipids, Alabaster, Ala.), or 250 nM N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-D-galactosyl-β1-1'-sphingosine (C6-NBD galactosyl ceramide or NBD-GalCer) (Avanti Polar Lipids). Aliquots (150 μl) were mixed with 150 μl HBSS containing 5 mg/ml fatty-acid free BSA (Sigma-Aldrich) and 500 nM Sytoxblue (Molecular Probes, Eugene, Oreg.), and analyzed by FACSAria.

Induction of Apoptosis

Apoptosis was induced with FasL as described (25). In brief, IFETs expressing mouse Fas were treated with 60 units/ml FasL at 37° C. for 2 h, and PS exposure was determined by flow cytometry with Cy5-Annexin V. To detect activated caspase 3, cells were fixed at 37° C. for 10 min in PBS containing 1% paraformaldehyde, permeabilized with 90% methanol at −20° C., and stained with rabbit mAb against active caspase 3 (Cell Signaling, Danvers, Mass.). Cells were then incubated with Alexa 488-labeled goat anti-rabbit IgG (Invitrogen), and analyzed by FACSAria.

Electrophysiology

TMEM16 sequences, FLAG-tagged at C-terminus, were inserted into pEF-BOS-EX (31). HEK293T cells (2.5×10^5) were co-transfected with 1.0 μg of TMEM16 expression vector and 0.1 μg of pMAX-EGFP (Lonza Group, Basel, Switzerland) using FuGENE6 (Promega, Madison, Wis.). At 24 h after transfection, cells were re-seeded on glass coverslips coated with fibronectin (Sigma-Aldrich). Within 24 h after re-seeding, whole-cell recordings of cells expressing EGFP were performed using a patch-clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, Calif.) as described (23,32). The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, and 10 mM Hepes-NaOH (pH 7.4). The intracellular solution contained 140 mM NaCl, **1.12** mM EGTA, **1** mM $CaCl_2$, 30 mM glucose, and 10 mM Hepes-NaOH (pH7.4). The free Ca^{2+}concentration (500 nM) was calculated with WEBMAXC software.

Results

Establishment of $TMEM16F^{-/-}$ Fetal Thymocyte Cell Lines

Ca^{2+}-dependent PS exposure is reduced by knocking down TMEM16F mRNA and accelerated by TMEM16F overexpression, suggesting that TMEM16F is a phospholipid scramblase (20). To demonstrate TMEM16F's involvement in Ca^{2+}-dependent phospholipid scrambling and to determine whether TMEM16F plays a role in exposing PS to the cell surface during apoptotic cell death, we established from fetal thymus tissue a TMEM16F-deficient mouse cell line that expresses a small number of TMEM16 family members, including TMEM16F (see below).

A targeting vector in which exon 2 of TMEM16F gene was flanked by loxP sequences was used to replace the TMEM16F allele in a mouse embryonic stem cell (ES) line from a C57BL/6 background (FIG. **1**A). Mice carrying the floxed allele were generated from the ES clone, and intercrossed. Embryos were genotyped at embryonic day 14.5, and fetal $TMEM16F^{flox/flox}$ thymus cells were infected with a retrovirus carrying $H\text{-}ras^{V12}$ and c-myc to establish IFET cell lines. Flow cytometry analysis showed that IFETs expressed Thy-1 weakly and CD44 strongly, but did not express CD4 or CD8; this indicated that they were derived from a T-cell lineage at an early developmental stage. A real-time RT-PCR analysis showed that IFETs expressed TMEM16F, 16H and 16K (FIG. **1**B). Next, IFETs were infected with adenovirus carrying the CRE recombinase

gene, and cells missing exon 2 of the TMEM16F gene were cloned (FIG. 1C). Removing exon 2 causes a frame-shift and truncates TMEM16F protein at the N-terminal region. Accordingly, Western blotting with an anti-TMEM16F antibody showed broad bands around 120 kDa in TMEM16F$^{flox/flox}$ but not TMEM16F$^{-/-}$ IFETs (FIG. 1D). An apparent Mr of TMEM16F detected by SDS-PAGE is slightly larger than the expected Mr for TMEM16F (106 kDa), which may be explained by glycosylation, since mouse TMEM16F carry 6 putative N-glycosylation sites (Asn-X-Ser/Thr).

Requirement of TMEM16F for Ca^{2+}-Induced, but not Apoptotic PS-Exposure

TMEM16F$^{flox/flox}$ IFETs treated at 20° C. with a Ca^{2+} ionophore A23187 quickly exposed PS (FIG. 2A); however, this exposure was completely absent in TMEM16$^{-/-}$ IFETs. Similarly, the treatment of TMEM16F$^{flox/flox}$ but not TMEM16F$^{-/-}$ IFETs with A23187 caused rapid PE-exposure, detected by binding of RO-peptide (20) (data not shown). We then examined the role of TMEM16F in lipid internalization, and found that TMEM16F$^{flox/flox}$ but not TMEM16$^{-/-}$ IFETs internalized NBD-PC and NBD-GalCer upon Ca^{2+}-ionophore treatment (FIGS. 2B and 2C). These results indicated that TMEM16F is responsible for Ca^{2+}-dependent lipid scrambling in IFETs.

In agreement with previous report showing that Fas is not expressed in T cells at early developmental stages (33), IFETs do not express Fas (FIG. 2D). When IFETs were transformed with mouse Fas, FasL efficiently activated caspase 3 (FIG. 2E) and the cells quickly responded by exposing PS (FIG. 2F). A TMEM16F-null mutation did not affect either FasL-induced PS exposure or caspase activation (FIGS. 2E and 2F). In cells undergoing apoptosis, cell size decreases and cellular granularity increases (34). Treating the TMEM16F$^{flox/flox}$ and TMEM16F$^{-/-}$ IFETs with FasL decreased the cell size (forward-scattered light, FSC) and increased the cellular granularity (side-scattered light, SSC) to the same extent (FIG. 2G). Therefore, we concluded that caspase-dependent apoptotic PS exposure and cell shrinkage take place independently of TMEM16F.

TMEM16 Family Members' Abilities to Expose PS

The ten TMEM16 family members have similar topologies, and 20-60% amino acid sequence identity (35,36). To examine TMEM16 family members' ability to scramble phospholipids, we transformed TMEM16F F$^{-/-}$ IFETs, in which the Ca^{2+}-dependent lipid scramblase activity is completely lost, with mouse retroviral vectors carrying FLAG-tagged TMEM16 family members. Since the expression plasmids for TMEM16C, 16D, and 16E with their endogenous sequences produced very low protein levels in IFETs, their sequences were modified to optimize the mRNA stability and translation efficiency. Western blots with an anti-FLAG mAb detected a specific band for each TMEM16 family member (FIG. 3A). Except for TMEM16K, their apparent Mr, detected by SDS-PAGE, is larger than the calculated Mr, which may be explained by glycosylation because these members carry 1-6 N-glycosylation sites. On the other hand, the apparent Mr (65 kDa) of TMEM16K, that does not have a putative N-glycosylation site, was significantly smaller than its estimated Mr (76 kDa). Some membrane proteins are known to behave anomalously in SDS-PAGE (37), and TMEM16K may belong to the group of this category. The Western blots also showed that most of the TMEM16 family members were expressed at similar levels, except that the TMEM16E level was 3-5 times lower, and TMEM16K level 5-10 times higher than those of other family members (FIG. 3A). As expected, Ca^{2+} ionophore treatment efficiently induced TMEM16F$^{-/-}$ IFET transformants expressing TMEM16F to expose PS (FIG. 3B). The TMEM16D—as well as TMEM16G and 16J—transformants also exposed PS upon Ca^{2+}-treatment, although the ability of TMEM16G, or 16J to enhance the PS exposure was weaker than that of TMEM16F and 16D. On the other hand, no or little PS-exposing activity was detected with TMEM16A, 16B, 16C, 16E, 16H and 16K. Similarly, TMEM16F$^{-/-}$ IFETs lost the ability to internalize NBD-PS, and this activity was rescued strongly by transforming the cells with TMEM16D, 16F, and 16J, and weakly by 16G. While, IFETs transformants expressing TMEM16C and 16E did not internalize NBD-PS (data not shown).

TMEM16 Family Members' Abilities to Scramble Lipids

TMEM16F scrambled not only PS and PE, but also other lipids (FIG. 2). To examine the lipid scramblase activity of other TMEM16 family members, TMEM16F$^{-/-}$ IFETs expressing TMEM16 family members were incubated with a fluorescent probe, NBD-PC or NBD-GalCer. As shown in FIG. 4A, the TMEM16F$^{-/-}$ IFETs expressing TMEM16D constitutively, or without A23187-treatment, internalized NBD-PC, and this internalization was strongly enhanced by the A23187 treatment. The A23187-induced NBD-PC uptake with the TMEM16D transformants was stronger than that observed with the 16F-transformants. Pre-treatment of TMEM16D-transformants with BAPTA-AM, a cell-permeable Ca^{2+} chelator, reduced the NBD-PC uptake observed without Ca^{2+}-ionophore (FIG. 4B), suggesting that the endogenous cellular level of Ca^{2+} is sufficient to activate the scrambling activity of TMEM16D. As with PS exposure, the A23187- treatment did not induce NBD-PC uptake in IFETs expressing TMEM16A, 16B, 16E, 16H, or 16K (FIG. 4A). However, cells expressing TMEM16C, 16G, or 16J did internalize NBD-PC when treated with Ca^{2+} ionophore.

A similar result was obtained using NBD-GalCer as a substrate. When treated with A23187, TMEM16F$^{-/-}$ transformants expressing TMEM16F incorporated NBD-GalCer, but those expressing TMEM16A, 16B, 16E, 16H, or 16K did not (FIG. 4C). Cells expressing TMEM16D constitutively incorporated NBD-GalCer, and this uptake was enhanced by A23187 treatment. The cells expressing TMEM16C, 16G, or 16J also internalized NBD-GalCer, although TMEM16C's ability to internalize NBD-GalCer was weaker compared with others. These results suggested that TMEM16C, 16D, 16F, 16G and 16J scramble various phospholipids and glycosphingolipids with some different substrate preference.

Chloride Channel Activity of TMEM16 Family Members

TMEM16A and 16B are Ca^{2+}-dependent Cl^- channels (22-24). To determine whether there are any other TMEM16 family Cl^- channels, and whether the scramblase activity of TMEM16 family members depends on Cl^--channel activity, human 293T cells were co-transfected with the TMEM16 expression plasmid and a vector expressing GFP (FIG. 5A). The Ca^{2+}-dependent chloride channel activity in GFP-positive cells was then determined by whole-cell patch clamp analysis (23). We chose 293T cell line as host cells because it has little Ca^{2+}-dependent Cl^--channel activity (FIG. 5B) and was used successfully to show that TMEM16A and 16B act as Cl^- channels (22-24).

In the patch-clamp analysis, increasing the intracellular free Ca^{2+} in the pipette solution to 500 nM yielded large outward rectifying currents in cells expressing TMEM16A or 16B (FIGS. 5B and 5C). In contrast, other TMEM16 family members induced little if any Ca^{2+}-dependent current in 293T cells, and the effect of increasing the pipette solution Ca^{2+} concentration from 500 nM to 5 μM was negligible (data not shown). Therefore, we concluded that within the TMEM16 family, only TMEM16A and 16B act as Ca^{2+}-

dependent Cl^- channels, and that the phospholipid scrambling activity of TMEM16C, 16D, 16F, 16G, and 16J is independent of Cl^--channel activity.

Expression of TMEM16 Family Members in Mouse Tissues

Real-time PCR analysis of TMEM16 mRNA in various mouse tissues showed that each tissue expressed only a limited number of TMEM16 family members (FIG. **6**). Of the two Cl^- channels of TMEM16 family, TMEM16A and 16B, we found that TMEM16B was strongly expressed in brain and eye tissues, but weakly expressed or absent in tissues where TMEM16A was strongly expressed, such as the pancreas, liver, salivary glands, stomach, lung, skin, and mammary glands. Of the 5 lipid scramblases of TMEM16 family, 16C, 16D, 16F, 16G and 16J, TMEM16F was ubiquitously expressed in various tissues. Whereas, other scramblases were present only in a few tissues: TMEM16C and 16J were strongly expressed in the brain and skin, respectively, while 16D was found at a low level in a few tissues such as the brain, ovary, heart, and eyes, and 16G and 16J were found in the stomach and intestines. Of the TMEM16 proteins that did not show scramblase or Cl^--channel activity, 16H and 16K were expressed ubiquitously in various tissues, while 16E was expressed only in the muscle and skin.

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The invention claimed is:

1. A method for screening an agent promoting or inhibiting a biological function of a transmembrane protein 16 (TMEM16) family member as a lipid scramblase, which comprises the following steps:

(1) treating TMEM16F deficient cells into which a gene encoding and expressing the TMEM16 family member selected from the group consisting of TMEM16C, TMEM16D, TMEM16G and TEMEM16J has been introduced with a candidate of the agent in the presence of Ca^{2+}, and

(2) determining whether the candidate alters distribution of a lipid selected from the group consisting of phosphatidylserine, phosphatidylcholine, and galactosylceramide in plasma membrane of the cells,

wherein a candidate which significantly increases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as an agent promoting a biological function of the TMEM16 family member as a lipid scramblase, and a candidate significantly which decreases distribution of phosphatidylserine in the outer leaflet of plasma membrane compared to control is selected as an agent inhibiting a biological function of the TMEM16 family member as a lipid scramblase, and

a candidate which significantly increases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as an agent promoting a biological function of the TMEM16 family member as a lipid scramblase, and a candidate which significantly decreases distribution of phosphatidylcholine or galactosylceramide in the inner leaflet of plasma membrane compared to control is selected as an agent inhibiting a biological function of the TMEM16 family member as a lipid scramblase; and

wherein

the cells are human, monkey, mouse, or rabbit cells,

the distribution of phosphatidylserine in plasma membrane is determined by detecting the binding between phosphatidylserine exposed to the cell surface and an agent having phosphatidylserine-binding property, and

the distribution of phosphatidylcholine or galactosylceramide in plasma membrane is determined by utilizing a fluorescently-labeled lipid.

2. The method of claim **1**, wherein the lipid is selected from phosphatidylcholine or galactosylceramide when the TMEM16 family member is TMEM 16C.

3. The method of claim **1**, wherein the lipid is selected from phosphatidylserine, phosphatidylcholine, or galactosylceramide when the TMEM16 family member is TMEM 16D.

4. The method of claim **1**, wherein the lipid is selected from phosphatidylserine, phosphatidylcholine, or galactosylceramide when the TMEM16 family member is TMEM 16G.

5. The method of claim **1**, wherein the lipid is selected from phosphatidylserine, phosphatidylcholine, or galactosylceramide when the TMEM16 family member is TMEM 16J.

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